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Evaluation of the presence of chlorogenic acids in coffee prepared by different processes

Dissertation for Master degree in Biological Engineering

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“Hardship often prepares an ordinary person for an extraordinary destiny.”

C. S. Lewis

Abstract

Chlorogenic acids (CGA) are water soluble phenolic compounds in coffee and have been found in several plants. They can be isolated from the leaves and fruit and they are the most abundant phenolic compounds in coffee. CGAs are subdivided according to the nature and number of cinnamic substituents and the esterification position in the cyclohexane ring of the quinic acid. CQAs are considered as the main isomer of CGAs in coffee. In the green beans of two main cultivated coffee species, Robusta and Arabica, CGAs account for 7-14.4% and 4-8.4% of dry matter basis (dm), respectively.

Considering the significant consumption of coffee beverages among European countries, and due to the contribution of CGAs to human health, a comprehensive study was performed to evaluate the content of CGA mainly: 3-caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA) and 4-caffeoylquinic acid (4-CQA), in coffee brews prepared by recent technologies. For this purpose, a method based on high performance chromatography – diode array detector (HPLC – DAD) was developed for the simultaneous determination of these three caffeoylquinic acids. To proceed to clean up of the extracted samples, Carrez solutions I and II were used for the precipitation of proteins, elimination of turbidity and breaking of emulsions.

A total of twenty-five coffee brews were prepared according to the manufacturers' instructions, considering coffee brews prepared with roasted and ground Arabica and Robusta coffee (boiled, French, mocha and filtered coffee), and seventeen types of commercial coffee brews prepared by different brewing techniques such as capsule, pod, espresso, instant, iced coffee and iced cappuccino, which were selected with regards to CQAs concentration.

The method presented good linearity with correlation coefficients greater than 0.99 and recoveries in the range of 91.0-103.5% for the three caffeoylquinic acids. The limits of detection were 0.37 mg/L for 3-CQA, 0.39 mg/L for 4-CQA and 0.18 mg/L for 5-CQA, while the limits of quantification were, 1.24 mg/L for 3-CQA, 1.29 mg/L for 4-CQA and 0.58 mg/L for 5-CQA.

Data indicated that the most abundant CQAs in all considered samples (except instant natural) were 3-CQA, followed by 5-CQA and 4-CQA. The decreasing order of total CQAs of samples from roasted and ground Robusta in normalized mg/L basis was mocha (872.93 mg/L) > boiled (771.29 mg/L) > French (666.67 mg/L) > filter (624.03 mg/L) and in brews prepared with Arabica were, 744.70 mg/L (boiled), 744.04 mg/L (mocha), 645.56 mg/L (French) and 638.58 mg/L (filter).

In the case of commercial coffees, the results varied according to the brewing processes and total CQAs ranged from 1662.01 mg/L in coffee pod, to 45.79 mg/L in iced cappuccino.

Keywords: Phenolic compounds, Chlorogenic acids, Caffeoylquinic acids, Coffee brews, Arabica coffee, Robusta coffee, HPLC-DAD.

Resumo

Os ácidos clorogénicos (CGA) são compostos fenólicos do café, solúveis em água, que podem ser encontrados em várias plantas, e isolados a partir das folhas e dos frutos. Estes consistem nos compostos fenólicos mais abundantes do café. Os ácidos clorogénicos são subdivididos de acordo com a natureza e o número de substituintes cinâmicos e a esterificação na posição do anel ciclo-hexano do ácido quínico. Os ácidos cafeoilquínicos são considerados os principais isómeros dos ácidos clorogénicos do café. Nos grãos de café provenientes das duas principais espécies cultivadas de café, Robusta e Arábica, os CGAs representam 7-14.4% e 4-8.4% de matéria seca (MS), respetivamente.

Considerando o consumo significativo de café na europa, e devido ao efeito dos CGAs na saúde humana, um estudo detalhado foi realizado para avaliar o conteúdo de CGAs em cafés preparados por diferentes técnicas, especialmente os isómeros: ácido 3-cafeoilquínico (3-CQA), ácido 5-cafeoilquínico (5-CQA) e ácido 4-cafeoilquínico (4-CQA). Com esta finalidade, um método baseado em cromatografia líquida de alta eficiência (HPLC - DAD), foi proposto para a determinação simultânea destes três ácidos cafeoilquínicos. Para proceder à limpeza das amostras extraídas, soluções de Carrez I e II, foram utilizadas.

Vinte e cinco amostras de café, preparadas por tecnologias diferentes, foram obtidas de acordo com as instruções dos fabricantes. Considerando bebidas preparadas com café Arábica e Robusta, torrado e moído (fervido, francêss, mocha e filtrado) e dezassete tipos de modos de preparação de café comerciais, tais como cápsulas, pod, espresso, café gelado instantâneo e cappuccino gelado.

O método apresentou uma boa linearidade, com coeficientes de correlação superiores a 0.99 e uma recuperação na gama de 91.0-103.5% para os três ácidos cafeoilquínicos. Os valores de limite de deteção obtidos foram, 0.37 mg/L para o 3-CQA, 0.39 mg/L para o 4-CQA e 0.18 mg/L de 5-CQA. Os valores de limite de quantificação foram, 1.24 mg/L para 3-CQA, 1.29 mg/L para o 4-CQA e 0.58 mg/L de 5-CQA.

Foi possível observar-se, através dos dados obtidos, que os CQAs mais abundantes em todas as amostras consideradas (exceto instantâneo natural) foram 3-CQA, seguido de 5-CQA e 4-CQA. A ordem decrescente de CQAs totais de amostras de Robusta torrado e moído foi mocha (872.93 mg/L)> fervido (771.29 mg/L)> francês (666.67 mg/L)> filtrado (624.03 mg/L) e em amostras preparadas com café Arábica foram, 744.70 mg/L (fervido), 744.04 mg/L (mocha), 645.56 mg/L (francês) e 638.58 (filtrado).

No caso de cafés comerciais, os resultados dos processos estudados variaram de acordo com os mecanismos de preparação, onde o total de CQAs variou de 1662.01 mg/L para pod a 45.79 mg/L para cappuccino gelado.

Palavras-chave: Compostos fenólicos, Ácidos clorogénicos, Ácidos cafeoilquínicos, Café, Café Arábica, Café Robusta, HPLC-DAD.

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And for all of you, who were part of this stage of my life:

"Friendship is unnecessary, like philosophy, like art… It has no survival value;
rather it is one of those things that give value to survival."

C. S. Lewis

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Glossary

3-CQA	3-caffeoylquinic acid
3-FQA	3-feruloylquinic acid
3-pCoQA	3-*p*-coumaroylquinic acid
3C	3-caffeoyl
3F	3-feruloyl
3,4-diCQA	3,4-diCaffeoylquinic acid
3,5-diCQA	3,5-diCaffeoylquinic acid
4-CQA	4-caffeoylquinic acid
4-FQA	4-feruloylquinic acid
4C	4-caffeoyl
4F	4-feruloyl
4-pCoQA	4-*p*-coumaroylquinic acid
4,5-diCQA	4,5-diCaffeoylquinic acid
5-CQA	5-caffeoylquinic acid
5-FQ	5-feruloylquinic acid
5-pCoQA	3-*p*-coumaroylquinic acid
ACN	Acetonitrile
C	Sample concentration
C1	Concentration level 1
C2	Concentration level 2
C3	Concentration level 3
CAS	Chemical Abstracts Service
CGA	Chlorogenic acid
CQAs	Caffeoylquinic acids
CoCQAs	Coumaroylcaffeoylquinic acids
CQA	Caffeoylquinic acid
DAD	Diode array detector
diCQA	DiCaffeoylquinic acid
Dm	Dry matter
EC	Espresso coffee
GC-MS	Gas chromatography – mass spectrometry
H	Value of the signal when the sample is analysed
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography – mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MAE	Microwave-assisted extraction
N	Noise ratio
NA	Not available
PDA	Photodiode array
R	Correlation of coefficient
S	Signal ratio
SPE	Solid phase extraction
UV	Ultraviolet

Chapter 1

Literature Review

1.1 Coffee: A perspective on processing

One of the most fascinating subjects for researchers throughout the world is coffee. Millions of people start their day with a cup of coffee. In Europe, Asia and Latin America, in busy and remote rural villages, this ritual is repeated providing enjoyment and sustenance (Nestlé, 2004). In 2012/13, 44 million (kilo bags) of coffee was consumed all over the world (International Coffee Organization, 2014). The main consumers were United States, Brazil, Germany, Japan, and Italy (Rodrigues & Bragagnolo, 2013). Coffee is appreciated because of its organoleptic characteristics (it produces pleasant taste and aroma) and stimulating and beneficial health effects (Rodrigues & Bragagnolo, 2013). The *Rubiaceae* family, to which coffee plant belongs, many species, but just seven of them are economically important (Ramalakshmi & Raghavan, 2003). Coffee beans belong to the *Coffea* genus (Crozier et al., 2012) where abundant commercially cultivated species are from the seeds of *Coffea Arabica* and *Coffea canephora* var *robusta* usually known as Arabica and Robusta coffee, respectively (Grembecka et al., 2007; Hečimović et al., 2011). The first one constitutes 65% of world's coffee productions, while 35% belongs to Robusta coffee (International Coffee Organization, 2014). Better sensorial properties of Arabica coffee cause its higher price in international market (Esquivel & Jiménez, 2012) while Robusta coffee considered to be more acidic (Mussatto et al., 2011), contains more caffeine and it is known because of its resistance to pests and disease (Ramalakshmi & Raghavan, 2003). Due to its high extractability of soluble solids, Robusta coffee is used usually for soluble coffee production (Rodrigues et al., 2013). However, different percentage of Arabica and Robusta coffee is considered for production of coffee blends with different qualities depending on consumer's preference (Grembecka et al., 2007).

There are several critical steps during the coffee processing procedure which affect the quality of coffee, namely: variety of coffee, environmental factors (soil, altitude), insect or fungal attack, method of processing, drying, hulling, and grading. Therefore, correct processing technique is found necessary in order to obtain high quality coffee (Ramalakshmi & Raghavan, 2003). A coffee tree takes on average 3 to 4 years to reach maturity and provide fruits. The coffee cherries develop slowly and become red at the end of a period of nine months (Nestlé, 2004). The coffee cherries have two coffee beans inside of them covered by a thin parchment like hull and further surrounded by pulp (Figure 1) (Mussatto et al., 2011; Nestlé, 2004). Coffee production process includes the removal of outer red skin and pulp from ripped coffee fruit followed by removal of mucilage, parchment covering and eventually the silver skin that covered

coffee beans (Spiller, 1998). The processing of coffee starts with the transformation of coffee cherries into green coffee beans. After harvesting, one of two alternative methods (wet or dry method) is used to separate skin, pulp and the parchment from them (Mussatto et al., 2011; Nestlé, 2004). The cherries are dried under sun heat or in a mechanical dryer in dry processing, commonly used for Robusta coffee (Mussatto et al., 2011). In this process the drying operation is the most important step because it affects the final quality of coffee (Duarte et al., 2010). In case of wet method, the skin and pulp are removed in a succession of mechanical and watery treatments (Nestlé, 2004). The parchment is removed by a hulling machine after the coffee dried (Mussatto et al., 2011; Nestlé, 2004). This process is generally used for Arabica coffee beans (Mussatto et al., 2011). The last step is to remove all the stones and other foreign matter in the hulled coffee beans (Nestlé, 2004). The result is considered as green coffee beans (Nestlé, 2004).

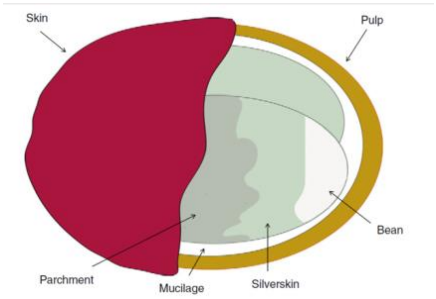


Figure 1 - Layers in a coffee fruit. Adapted from Esquivel & Jiménez (2012)

The most important step for producing the flavour, aromatic compounds and colour in coffee is the roasting step, which affects the quality of the coffee beverage (Mussatto et al., 2011). In this process the plant materials will suffer the thermal transformation resulting on the coffee bean roasting (Spiller, 1998). From the technical point of view roasting is a very complex process. During the roasting, moisture loss and different changes in colour, volume, mass, form, pH, density and volatile components occur and CO_2 is generated. The time-temperature of this process leads to several chemical reaction (oxidation, pyrolysis, degradation, dehydration, hydrolysis, etc.) and produce Maillard reaction products and various organic compounds (Mussatto et al., 2011) representing the characteristic flavour of coffee (Ayelign & Sabally, 2013). After roasting process, coffee beans should be rapidly cooled in order to stop exothermic reactions and to prevent excessive roast (Mussatto et al., 2011). After a final quality control, the ground coffee is vacuum sealed and shipped, then distributed to coffee shops and retailers (Mussatto et al., 2011; Nestlé, 2004). The process follows with storage of coffee that will be traded in market to go to the factory to be grounded (Nestlé, 2004).

1.2 Brewing techniques

The technological operation of preparation of drink coffee is called brewing, which may be prepared at domestic or catering level. In fact brewing is a kind of solid-liquid extraction, by

which roasted and ground coffee is mixed with hot water (Nicoli et al., 2010). Under brewing, soluble volatile substances, such as pyrazynes, aldehydes, ketones (responsible for coffee aroma) and not volatile substances like caffeine, acids, sugar (responsible for the taste of coffee) along with emulsifiable compounds such as protein, lipids, polysaccharides (responsible for body and foam formation) it were extracted to the surrounding water (Albanese et al., 2009).

Regardless of soluble coffee, extraction methods may vary from country to county based on consumers' preferences and are divided in three main categories, namely: decoction (boiled, Turkish, percolate coffee), infusion (filter and napoletana coffee) and pressure methods (French press, mocha and espresso) (Petracco, 2001). Each process produces distinct types of beverages (Lima, 2008) that influences the brew's chemical composition.

The most basic brewing technique seems to be boiled coffee where roast and ground coffees are boiled on a stove (Petracco, 2001). Another process is the mocha coffeemaker that is much extended at the domestic level in southern European countries such as Italy and Spain (Lima, 2008). Autoclave-type steel kettle led to water heats more than 100 ºC and the produced pressure force water through the coffee cake (Figure 2) (Petracco, 2001).

The filtration – percolation method is done using a coffee pot where the filter is clear and bright. The contact time between ground coffee and water is limited being necessary a fine grinding so that the extraction occurs by slow gravity percolation (Lima, 2008; Mussatto et al., 2011). Metal, synthetic or paper filters can be used for brew preparation. The plastic filters, conical or basket, exist in different sizes corresponding to the cups number to be prepared (Lima, 2008).

The French press simple method does not require the use of a strainer and it is easier to calculate the right amount of water (Figure 2). A fine grinding may hinder the movement of the piston that separates the coffee powder ready from the extract and also have sediments at the bottom of the cup (Lima, 2008).

The espresso coffee (EC) is the most common coffee in Portugal and it has specific aroma characteristics topped with *crema* (espresso foam). Different methods are used to make espresso coffee and the most common are with bar machines, capsules and pod. Nowadays, capsule and pod are one of the most homemade espresso coffees with a big commercial popularity due to their using friendly characteristics as well as high quality of the resulted brew which leads to design them in large numbers. The bar machines are the conventionally brewed which consist of a rotating pump, a heat exchanger and an extraction chamber (Parenti et al., 2014). EC is the coffee brewing technique based on pressure, where the water is hot forced (90±5 ºC) under desired pressures (9±2 atmosphere) and pass through the roasted and ground coffee (6.5±1.5 g) to produce a small cup of concentrated beverage during short time (30±5 s) (Petracco, 2001). Tips of EC preparation are well documented by Illy and Viani (2005) and Petracco (2001). Final

quality of EC may vary from one cup to another one, depending on type of coffee, roasting degree (Nunes et al., 1997), water quality (Navarini and Rivetti, 2010) and as well as on personal preferences (Illy and Viani, 2005). Some brewing techniques are shown in Figure 2.

Mocha pot French press Espresso

Figure 2 - Brewing principles for some types of coffee. Adapted from Food&Wine (2013), CoffeeGeek (2004), GallaCoffee (2014).

In case of soluble coffees, a longer manufacturing process is involved and several extraction and drying processes are needed to obtain soluble coffee powder. Instant coffee production involves ground-roast treatment with hot water and high pressure extracting the water-soluble compounds. After this treatment, the coffee is then cooled, centrifuged, concentrated by heating and dried by freeze-drying that uses very low temperatures. The spray-drying process uses high temperature under high pressure to volatilize the aqueous extract (Farah, 2012). Since seeds contain higher amounts of soluble solids led to increasing yield, in blends designated for instant coffee production, generally, Robusta coffee is used (Farah, 2012).

Regarding cup size of coffee brews, great variability observe among a size of single serving, ranging from 15 mL related to concentrated Italian espresso to over 250 mL in many English-speaking countries which may obtain from brewing of 5 g to more than 15 g roasted and ground coffee (Petracco, 2005).

1.3 Chemical composition of coffee

From a chemical point of view, the main coffee species (Arabica and Robusta) present a rich source of biologically active compounds like lipids, caffeine, chlorogenic acids, amino acids, cellulose, mineral salts, sugars and niacin and because of that it is considered a nutraceutical food (nutritional and pharmaceutical) (Table 1) (Ayelign & Sabally, 2013). Keeping in mind that the coffee species (Arabica or Robusta) are known to have great effects on chemical composition of beans, however, the effect of other parameters like genetic aspects, soil-climatic conditions, agricultural practices, post-harvest techniques (Joët et al., 2010) as well as the degree of coffee fruit maturation and contamination during harvesting should also be considered, although they affect chemical composition to a lesser extent. Regarding coffee brews, differences in coffee species (Alves et al., 2010; Vignoli et al., 2011) coffee/water ratio (Andueza el al., 2007; Buchmann et al., 2009), particles size (Andueza, De Peña, & Cid, 2003; Buchmann et al., 2009), extraction temperature (Buchmann et al., 2009; Andueza et al., 2003; Caprioli et al., 2013) or water pressure (Andueza et al., 2002; Caprioli et al., 2013) result in a great chemical diversity or different sensorial properties in coffee brew.

Chemical composition of green beans consists in non-volatile and volatile compounds. The non-volatile compounds are composed by water, carbohydrates and fiber, proteins and free amino acids, lipids, minerals, organic acids, chlorogenic acids, trigonelline and caffeine. Chemical composition of green and roasted Arabica and Robusta coffee can be found in Table 1. The most abundant classes of volatile compounds are alcohols, esters, hydrocarbons, aldehydes, ketones, pyrazines, furans and sulfur, seem to be related to the maturation stage (Farah, 2012). Among chemical compounds in coffee, lipids, chlorogenic acids and caffeine are of interest not only because of their quantity but also due to their potential effect on human health (Li et al., 2014; Boekschoten et al., 2003; Nawrot et al., 2003).

Caffeine is classified as a methylxanthine alkaloid with bitter characteristics and it is a central nervous system stimulant (Yassin, 2008). This alkaloid is a thermostable compound and it is not destroyed under excessive roasting conditions (Mussatto et al., 2011; Farah, 2012) and its concentration in Robusta coffee is twice than in Arabica coffee (Farah, 2012). The effect of brewing mechanisms on caffeine content of various type of coffee brew was investigated previously (Bell et al., 1996). According to Bell et al. (1996) higher amounts of coffee grounds and volumes of coffee, caused considerable increase on caffeine yields.

Chlorogenic acids comprise a major class of phenolic compounds and are well explained in next section. The higher chlorogenic acids content, as it is found in Robusta coffee, protect the plant against microorganisms, insects and UV radiation but may reduce cup quality whereas low amounts of chlorogenic acids contribute to coffee flavour (Farah, 2012). The chlorogenic acids in coffee beans are present in proportions that may vary from 7% to 12% (w/w) even in Robusta or Arabica coffee, three to five times more than the caffeine (Mussatto et al., 2011).

One of the most important constitute in coffee is lipid varies considerably between Arabica and Robusta coffee, accounts for approximately 7-17% (w/w) of coffee beans (an average amount of 15% and 10% for green Arabica and Robusta coffee, respectively). Green coffee beans are composed mainly by triacylglycerols (75.2%), esters of diterpene alcohols and fatty acids (18.5%), free diterpene alcohols (0.4%), esters of sterols and fatty acids (3.2%), sterols (2.2%), tocopherols (0.04-0.06%), phosphatides (0.1-0.5%) and tryptamine derivatives (0.6-1.0%) (Speer & Kölling-speer, 2006). Carbohydrates are another major constituent of coffee and may account for more than 50% of the dry weight. Polysaccharides account for 44% of dry matter in Arabica coffee and 47% in Robusta. Actually, Robusta coffee contains more soluble solids whereas Arabica coffee provides superior cup quality and aroma compared with Robusta (Farah, 2012). Protein, peptides and free amino acids are needed for de Maillard reaction, they serve as precursors for the formation of volatile compounds (Farah, 2012).

Table 1 - Chemical composition of green and roasted Arabica and Robusta coffee. Adapted from Farah (2012) in accordance with Iarc (1989)

*Compounds**	*Green Coffee Beans (g/100g)*		*Roasted Coffee Beans (g/100g)*	
	Arabica coffee	*Robusta coffee*	*Arabica coffee*	*Robusta coffee*
		Carbohydrates/fiber		
Sucrose	6.0-9.0	0.9-4.0	4.2	1.6
Reducing sugars	0.1	0.4	0.3	0.3
Polysaccharides	34-44	48-55	31-33	37
Lignin	3.0	3.0	3.0	3.0
Pectin	2.0	2.0	2.0	2.0
		Nitrogenous compounds		
Protein	10.0-11.0	11.0-15.0	7.5-10	7.5-10
Free amino acids	0.5	0.8-1.0	ND**	ND
Caffeine	0.9-1.3	1.5-2.5	1.1-1.3	2.4-2.5
Trigonelline	0.6-2.0	0.6-0.7	0.2-1.2	0.3-0.7
Nicotinic acid	-	-	0.016-0.026	0.014-0.025
		Lipids		
Coffee oil	15-17.0	7.0-10.0	17.0	11.0
Diterpene esters	0.5-1.2	0.2-0.8	0.9	0.2
		Minerals		
Minerals	3.0-4.2	4.4-4.5	4.5	4.7
		Acids and esters		
Chlorogenic acids	4.1-7.9	6.1-11.3	1.9-2.5	3.3-3.8
Aliphatic acids	1.0	1.0	1.6	1.6
Quinic acid	0.4	0.4	0.8	1.0
Melanoidins	-	-	25	25

* Content varies according to cultivar, agricultural practices, climate, soil composition, methods of analysis, and roasting degree.
**Not Detected

Regarding other compounds, at least 14 elements both in Arabica and Robusta were recognized in literatures (Debastiani, 2012; Farah, 2012). Among the analysed elements are magnesium, phosphorus, potassium, calcium, manganese, iron, copper and zinc, where potassium accounts for approximately 40% of the mineral content of coffee (1-2 g/100 g green coffee) (Debastiani, 2012). Among them, only magnesium seems to vary considerably between species (1-3 mg/100 g for Robusta and 2.5-6 mg/100 g for Arabica) (Farah, 2012).

1.4 Chlorogenic acids

Phenolic compounds are constituents of plant foods such as fruits, vegetables, cereals and legumes, and beverages of plant origin, such as wine, tea and coffee (Farah & Donangelo 2006; Olthof et al., 2001). Phenolic compounds can be grouped into different classes according to their basic chemical structure (type and number of phenol rings), and into different subclasses (Farah & Donangelo, 2006). Among them, Chlorogenic acids (CGA) are the most abundant phenolic compounds in coffee (Farah & Donangelo, 2006). They are subdivided according to the nature and number of cinnamic substituents and the esterification position in the cyclohexane ring of the quinic acid (Farah, 2012). These compounds may be presented in monoester or diester with quinic acid (Ayelign & Sabally, 2013; Caprioli et al., 2013; Farah et al., 2008; Farah & Donangelo 2006).

The main phenolic compounds in coffee derived from trans cinnamic acid are caffeic (3-4-dihydroxy-cinnamic acid), ferulic (3-metoxy, 4-hidroxy-cinnamic acid), and *p*-coumaric (4-

hidroxy-cinnamic acid) acids. Naturally, they may be presented in monoester or diester with quinic acid and then called chlorogenic acids (Ayelign & Sabally, 2013; Caprioli et al., 2013; Farah et al., 2008; Farah & Donangelo 2006). Quinic acid has axial hydroxyl groups on carbons 1 and 3, and equatorial hydroxyls on carbons 4 and 5. Esters of this acid are formed on carbon 5, 3 and 4, and less commonly on carbon 1 (Farah & Donangelo, 2006).

CGAs are water soluble compounds (Rodrigues, 2013) and include mainly, caffeoylquinic acids (CQA, monoester of caffeic and quinic acid), dicaffeoylquinic acids (diCQA, diester of caffeic and quinic acid), feruloylquinic acids (FQA, monoester of ferulic and quinic acid), and *p*-coumaroylquinic acids (*p*CoQA, monoester of *p*-coumaroylquinic and quinic acid) (Farah & Donangelo, 2006).

Green coffee beans contain various groups of CGA as follows: CQAs with 3 isomers (3-, 4- and 5-CQA); FQA with 3 isomers (3-, 4- and 5-FQA); diCQA with 3 isomers (3,4-diCQA; 3,5-diCQA; 4,5-diCQA); *p*CoQA with 3 isomers (3-, 4- and 5-*p*CoQA), and as well as six mixed diesters of caffeoyl-feruloyl-quinic acids (CFAQ) (Crozier et al., 2012; Dokli et al., 2013; Farah & Donangelo 2006). CQAs are considered the main isomer of CGAs in coffee as 5-CQA alone is responsible for about 56–62% of total CGA followed by 3-CQA and 4-CQA. The second important group of isomers are diCQAs account for aproximately 15–20% of total CGA followed by FQA (5-13%) (Farah & Donangelo, 2006). Table 2, represent the chemical structure and characteristics of CGAs found in coffee.

In the green beans of two main cultivated coffee species, Robusta and Arabica, CGAs account for 7.0-14.4% and 4.0–8.4% of dry matter basis (dm), respectively (Farah & Donangelo, 2006). Nevertheless, some authors indicated that epresso coffees prepared with Arabica coffee were richer in terms of CGAs than brews prepared with Robusta coffee (Caprioli et al., 2013).

There are several procedures in coffee production which may influence the CGAs content in final product, such as fermentation, bean roasting, freeze or spray drying (in case of instant coffee) and decaffeination or blending (Mills et al., 2013). Among these, the roasting has the most profound effect on the chemical composition of coffee (Mills et al., 2013). Consequently, the CGAs content in brewed coffee may change due to numerous parameters like coffee species, roasting degree (Farah et al., 2005; Moon et al., 2009) origin of beans (Campa et al., 2005) and subsequent brewing methods (Fujioka and Shibamoto, 2008; Tfouni et al., 2014).

During roasting process, chlorogenic acids reducing up to 90% occurs and by the loss of a water molecule from the quinic acid moiety and formation of an intra-molecular ester bond, corresponding chlorogenic acid lactones are formed (Farah et al., 2005; Rodrigues & Bragagnolo, 2013). The effects of roasting on total chlorogenic acid content of coffees from different origins are presented in Figure 3.

Table 2 - Characteristics and structure of the main groups of compounds of chlorogenic acid in coffee beans*

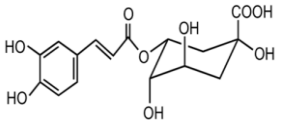
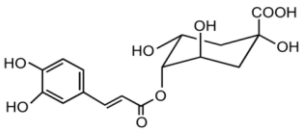
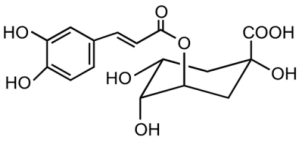
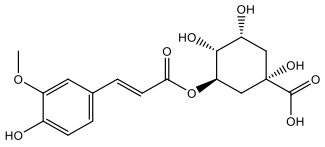
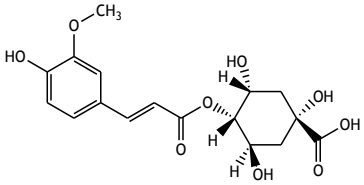
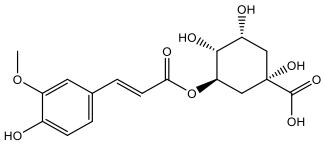
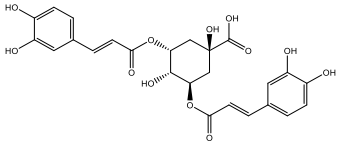
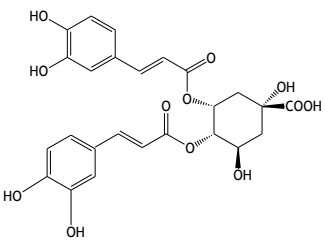
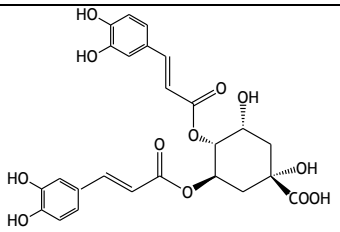
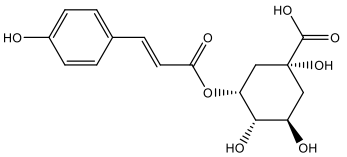
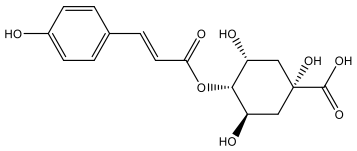
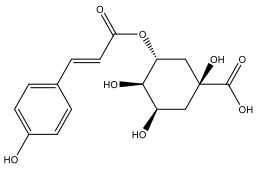
Groups of compounds	*Synonym*	*CAS number*	*Molecular Formula*	*Structure*	*Molecular weight*
			Caffeoylquinic Acids (CQAs)		
3-Caffeoylquinic acid	Chlorogenic acid	327-97-9	$C_{16}H_{18}O_9$		354.31
4-Caffeoylquinic acid	Cryptochlorogenic acid	905-99-7	$C_{16}H_{18}O_9$		354.31
5-Caffeoylquinic acid	Neochlorogenic acid	906-33-2	$C_{16}H_{18}O_9$		354.31
			Feruloylquinic Acids (FQAs)		
3-Feruloylquinic acid	-	NA**	$C_{17}H_{20}O_9$		368.33
4-Feruloylquinic acid	-	2613-86-7	$C_{17}H_{20}O_9$		368.33
5-Feruloylquinic acid	3-O- Feruloylquinic acid	1899-29-2	$C_{17}H_{20}O_9$		368.33
			diCaffeoylquinic Acids (diCQAs)		
3,5-diCaffeoylquinic acid	Isochlorogenic acid A	2450-53-5	$C_{25}H_{24}O_{12}$		516.45
3,4-diCaffeoylquinic acid	Isochlorogenic acid B	14534-61-3	$C_{25}H_{24}O_{12}$		516.45

Table 2- Characteristics and structure of the main groups of compounds of chlorogenic acid in coffee beans (cont.)*

Groups of compounds	*Synonym*	*CAS number*	*Molecular Formula*	*Structure*	*Molecular weight*
4,5-diCaffeoylquinic acid	Isochlorogenic acid C	32451-88-0	$C_{26}H_{26}O_{11}$		514.48
	p-Coumaroylquinic Acids (p-CoQAs)				
3-*p*-coumaroylquinic acid	-	1899-30-5	$C_{16}H_{18}O_8$		338.31
4-*p*-coumaroylquinic acid	-	93451-44-6	$C_{16}H_{18}O_8$		338.31
5-*p*-coumaroylquinic acid	-	NA	$C_{16}H_{18}O_8$		338.31

*This information was adapted from Fujioka & Shibamoto, 2008 and PubChem, 2014; **Not Available (NA).

When considering the technological properties, CGAs contributes to the formation of pigments, taste and flavour of coffee beans determining the quality of the beverage (Ayelign & Sabally, 2013; Caprioli et al., 2013).

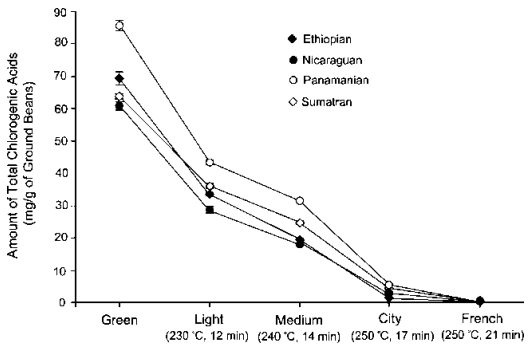


Figure 3 - Chlorogenic acids content changes in coffee beans during roasting process. Adapted from Moon et al. (2009).

Several authors have suggested a relationship between the composition of the CGAs fraction and the quality of the beverage; addition of dicaffeoylquinic acids was negative for coffee flavour, whereas addition of mono caffeoylquinic acids brought positive results (Ohiokpehai et al., 1982). These compounds are known to contribute to the final acidity and confer astringency and bitterness to the beverage (Ayelign & Sabally, 2013). The astringency of dicaffeoylquinic acid (diCQA) was investigated by Clifford & Ohiokpehai, (1983) and Naish, Clifford, & Birch, (1993) and the same response was observed for 5-CQA, tannic acid and grape seed tannin, which traditionally are associated to astringency. The bitterness will increase during roasting as a result of Maillard and Strecker's reactions (Farah & Donangelo, 2006).

Many researches have been undertaken and indicated the antioxidant and anti-inflammatory properties of CGAs (Ayelign & Sabally, 2013; Caprioli et al., 2013; Farah et al., 2008; Farah, 2012). They are associated with reduction of the relative risk of cardiovascular disease. Besides that, they also possess protective effects against type 2 diabetes and Alzheimer's disease (Kim et al., 2012; Rodrigues, 2013; Thom, 2007). In addition, they have exhibited hypoglycemic, antiviral, hepatoprotective and antispasmodic activities (Farah et al., 2008).

The daily intake of CGAs by coffee drinkers is considered to be 0.5–1.0 g (Johnston et al., 2003;Thom, 2007). It is also known that a diet rich in CGAs compounds plays a great role in preventing various diseases associated with oxidative stress such as cancer, cardiovascular, aging and neurodegenerative disease (Farah et al., 2008).

1.4.1 Methods for analysis of chlorogenic acids in coffees

In this section the more usual methods used for the determination of CGAs, mainly CQAs in coffee matrices, beans and brews, are presented.

The experimental procedures to evaluate CGAs contents in coffee beans are commonly performed by extraction and subsequent purification, to obtain a clear extract containing CGAs with less interfering compounds (Ky et al., 1997). The analysis of individual isomers has been carried out mainly by HPLC-DAD (Farah et al., 2005; Tfouni et al., 2014; Mills et al., 2013). Quantification of chlorogenic acids content in large populations of coffee beans needs an accurate, fast, and unbiased purification method. Usually, prior the HPLC analysis, sample preparation is required to remove some compounds that may interfere with the detection of target compounds, reducing the separation efficiency or the column life. Depending on the type of matrix, coffee beans or brews, different types of extraction and purification may be used.

1.4.1.1 Extraction and purification techniques

The basic technique for the extraction of bioactive compounds is solvent extraction which may be influenced by various parameters like type of solvent and its polarity, particle size of samples and extraction procedures (Taha et al., 2011). Solvent extraction has disadvantage of long extraction time which might lead to thermal degradation of the phytoconstituents. The extracting solvents used for phenolic compounds are methanol, ethanol, acetone, water, ethyl

acetate and to a lesser extent, propanol, dimethyl formamide and their combinations. The choice of the solvent, the use of heat, agitation ant time will affect solubility and mass transfer of compounds. Critical points in solvent extraction techniques are mainly the correct choice of solvents and the use of heat and agitation to increase the solubility of the desired compounds and improve their mass transfer (Taha et al., 2011).

With regards to CGAs, different extraction methodologies have been proposed for estimation of these compounds in coffee beans which basically are almost the same. Usually samples were extracted in a mixture of water-methanol followed by filtration. However, this technique is time consuming and requires successive amount of solvent for each experiment. Concerning coffee beans, energy-consuming and laborious efficient extraction of these compounds and production of concentrated extract without interfering compounds from coffee beans are the main difficulties in analysis of these compounds.

Novel extraction methods like microwave assisted extraction (MAE), supercritical fluid extraction and ultrasound assisted extraction use shorter extraction time and thus show a reduced solvent consumption and protect thermolabile constituents (Taha et al., 2011).

The most potential alternative to conventional solvent extraction is microwave assisted extraction (MAE). Feasibility of applying MAE conditions to maximize CGAs extraction under different conditions was investigated by Upadhyay et al. (2012). MAE is a process that uses microwave energy, along with solvent, to extract target compounds resulting in reduced time and solvent consumption. This process shows higher yields under optimum conditions than the conventional solvent extraction and can be predicted and controlled for industrial application (Upadhyay et al., 2012). The values obtained for CGAs extraction yields revealed the significantly higher and better results for MAE than the ones of the conventional heat reflux method (Table 3) (Upadhyay et al., 2012).

Table 3 - Comparative analysis between conventional heat reflux and microwave assisted to extract CGAs from Robusta green coffee beans. Adapted from Upadhyay et al. (2012)

Method of extraction	*Parameters*	*% Yield (Chlorogenic acid)*
Conventional heat reflux method of extraction	Time (5 min), Temperature (50 ℃), Sample: solvent (1:4)	3.95±0.21
Microwave-assisted extraction	Time (5 min), Temperature (50 ℃), Wattage (800 W), Sample: solvent (1:4)	8.40±0.28

The ultrasound assisted extraction is an inexpensive, simple and efficient alternative compared to conventional extraction techniques. The main advantages include the increase of extraction yield and faster kinetics (Tadeo et al., 2010). By the use of sonication, the operating temperature can be reduced, allowing the extraction of temperature-sensitive components. Compared with other novel extraction techniques such as MAE, the ultrasound apparatus is

cheaper and its operation is easier (Tadeo et al., 2010). Some research has been published on ultrasound assisted extraction of CGAs from coffee samples (Tfouni et al., 2014). Although recently, numerous techniques are proposed for extraction of CGAs from coffee beans, many authors used the conventional solvent extraction, probably due to simplicity of the method.

Extraction of CGAs from green coffee beans in a mixture of methanol/water (70/30) containing 0.5% Na_2SO_3 followed by filtering through cotton filter for elimination of powder was proposed by Ky et al. (1997) which by its turn adopted from Colonna, (1979).

The method described by Farah et al. (2005) involves two simple steps. First, ground coffee was suspended in aqueous methanol (40%) and shaken at room temperature and after filtration the residue was washed again with water and the clear extract was subjected to purification.

Moon et al. (2009) also suggested the similar pattern, but in two different solvents. The approach consists of the use of 50 mL of hot water (85 ºC) or methanol/water (7/3). The ground coffees were soaked in hot water or the mixture of methanol/water and stand at room temperature for duration of 3 and 7 hours, respectively. They indicated that methanol/water led to higher recovery of CQAs up to 40% than the extract prepared using hot water.

Interfering compounds removal from extracts (extract of beans or the coffee brew) is a crucial step for estimation of CGAs. Different procedures were described in the literature (Ky et al., 1997) for purification of coffee extract.

In 1984, Trugo and Macrae, purified the methanol extract of coffee beans, directly with Carrez reagents (I and II) where solution I is prepared by dissolving crystalized zinc acetate (21.9 g) and glacial acetic acid (3 mL) in distilled water and solution II consists in potassium hexacyanoferrate (10.6 g) in 100 mL of distilled water, without previous evaporation of methanol in a rotary evaporator. Besides that, rapidity, less solvent consumption, acceptable repeatability and precision were some of the advantages of this method.

Other technique used for the purification of coffee bean extract was based on precipitate of proteins followed by lipids, pigment and caffeine removal from the aqueous extract, after evaporation of methanol from extract (Rakotomalala, 1992). Successive use of different organic solvent was one of the disadvantageous of this method. In terms of total CGAs, the method resulted in lowest concentration than other purification methods due to the loss of compounds during the different step of purification (Ky et al., 1997). Therefore, development of a new technique for the purification of chlorogenic acid was found essential.

DIN Standard 10767 (1992) describes an environmentally friendly alternative methodology based on directly analysis of methanol extract of coffee bean. The absence of Carrez reagents decreased recovery in the case of coffee beans due to the reaction of polysaccharides, soluble proteins, and other colloidal materials with CGAs (Ky et al., 1997). Crozier et al. (2013) applied this method to coffee brews for determination of CGAs. Diluted coffee brew was prepared with methanol and directly injected for HPLC-PDA analysis.

Another approach is the application of solid phase extraction (SPE). Bicchi et al. (1995) described a method based on purification of aqueous extract of coffee bean (without methanol) on C18 cartridge eluted with methanol/water (70/30) followed by analysis with HPLC-UV. The

precision of the following method was clearly low especially for FQAs and diCQAs (Ky et al., 1997). This method was further used by Caprioli et al. (2013) for purification of coffee brew (Caprioli et al., 2013). The method was validated through different validation parameters resulted in precision less than 5%. The obtained LOD and LOQ, for 5-CQA, was 0.08 and 0.25 mg/L, respectively. The LOD (0.1 mg/L) and LOQ (0.3 mg/L), for 3-CQA, was acceptable. Recovery was performed and varied between 67 to 97% (lower recovery for 3-CQA) seems to be because of sample loss during the purification.

In brewed coffee solid-phase extraction can be used in order to extract the solute from the liquid phase or solid phase. SPE is a good method because each step can be controlled. The selection of the elution liquid lets to ensure that the final extracts have the desire purity (Simpson, 2000). This method has the disadvantage of being time-consuming and using large amounts of organic solvents (Simpson, 2000).

At the same time, Balyaya and Clifford (1995) suggested treating the aqueous extract of coffee with Carrez reagents (solutions I and II). This method resulted in lower repeatability and less CGAs content compared to the previously reported method (Trugo and Macrae, 1984).

Ky et al. (1997) compared different procedures to find the fastest, most accurate, and the least biased methodology for purification of coffee extract and they found the method of Trugo and Macrae (1984) as an acceptable procedure for routine analysis of CGAs in coffee beans. According to the authors' knowledge, a significant number of reports have followed the method of Trugo and Macrae (1984) with minor modification in order to purify the coffee beans extract (Farah et al., 2005; Farah et al., 2006; Moon et al., 2009; Duarte et al., 2010; Monteiro & Farah, 2012).

1.4.1.2 Instrumental methods to determine chlorogenic acids

Several instrumental methods have been proposed for the identification and quantification of CGAs in coffee (Caprioli et al., 2013). The simplest methods for the analysis are based on ultraviolet absorption of alcoholic extracts or colorimetric methods. These methods show lack of specificity even using differential colorimetric techniques due to interferences with individual isomers (Trugo & Macrae, 1984).

The chromatographic techniques have greatly improved the precision of analytical data and, even though gas chromatography provides excellent resolution, HPLC (high-performance liquid chromatography) is preferred and it is the most common method, coupled with diode array detector (DAD) (Figure 4), as it avoids the degradation of compounds during the analysis (Caprioli et al., 2013; Trugo & Macrae, 1984). DAD consists in an incorporation of large number of diodes making possible simultaneous monitoring of more than one absorbing component at different wavelengths (Bhanot, 2012). These detector benefits are based on saving time and cost

reduction on expensive solvents (Bhanot, 2012). HPLC has the ability to separate and quantify the compounds that are present in any sample that can be dissolved in a liquid (Waters, 2013).

Other procedures, such as the simultaneous determination of total CGAs and caffeine in coffee by high performance gel filtration (HPGF) chromatography, have also been reported (Maria, 1995). Based on this method, it was achieved a good recovery with good correlation coefficients. This method appears to be useful for fundamental investigation and quality control in the industry (Maria et al., 1995).

Chromatography is a physical method of separation of substances, in which the separation of the components to be analysed is based on differential partitioning between a mobile phase and a stationary phase held in a column. When coupled with mass spectrometry (GC-MS), this analytical method allows, not only the separation of the components of a mixture, but also the characterization of those components (Skoog, 2007).

To proceed to the identification of chlorogenic acids it is primarily done a comparison with retention time of the respective standards and by spiking samples with small amounts of the appropriate standards (Farah et al., 2006). This aspect can be confirmed by LC-MS (Farah et al., 2006). The electrospray ionization source is operated in the negative mode to generate [M – H]- ions and in the single ion monitoring mode to detect chlorogenic acids specific mass ions (Farah et al., 2006).

Liquid Chromatography (LC), combined with mass spectrometry (MS), during the last decade has become a powerful analytical tool. Today, LC-MS has evolved into a technique characterized by sensitivity, selectivity, and specificity, allowing for the analysis of trace amounts of target analytes in complex mixtures. Sample preparation prior to analysis could be minimized or even eliminated (Dams & Huestis, 2003).

One limitation associated with LC-MS analysis is its susceptibility to matrix effect that consists in the effect of co-elution residual matrix components on the ionization of the target analyte. Matrix effect thus limits the utility of LC-MS for quantitative analysis (Dams & Huestis, 2003).

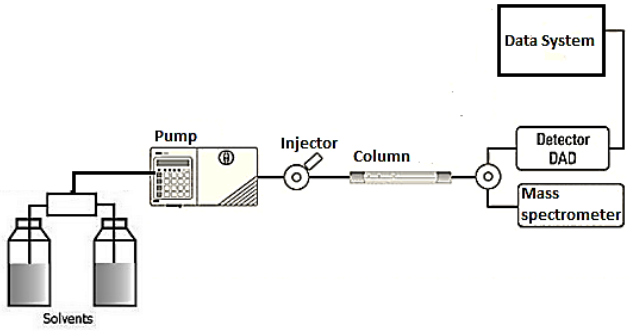


Figure 4 - Scheme of a typical HPLC-DAD-MS system. Adapted from Baskerville (2011).

1.5 Thesis organization

This thesis is outlined in several sections. In the first part a short introduction regarding coffee bean processing and different brewing procedures was presented. Subsequently chemical composition of coffee, in particular, chlorogenic acids as well as their subsequent impact on coffee quality and human health were monitored in order to represent the importance of investigation of respective compounds. Besides that, extraction techniques along with analytical methods for quantification of chlorogenic acids were reported. A comprehensive summary of the published work about chlorogenic acids was carried out in State of the Art in section 2, where subsequent concentrations of CGAs in beans and brews were discussed. The section 3, entitled Technical Description, includes materials and methods used to study the chlorogenic acids in various types of coffee brews. Afterwards, section 4, as it is the most important part, was presented as Results and Discussions, where the results achieved were reported and discussed, extensively. Section 5 is related to the main conclusions and the limitations of the present project, respectively. Appendix includes the additional information of the respective study.

Although the CQA concentration as the most abundant derivative of CGAs in coffee beans and the effect of processing conditions especially roasting on CGAs were frequently reported by other authors, data relating to the influence of other processing, mainly brewing procedures are limited. Besides that, few research papers reported the validation of their analytical methods with regards to CGAs.

Chapter 2

State of the Art

Coffee can be brewed in many ways depending on consumers' preference but recently consumer choices for a particular type of coffee beverage have been affected by various parameters. They prefer to know more about the chemical composition of their coffee brew and the potential impact of specific compounds on their health so coffee beverages have received a great deal of attention due to their high consumption and its subsequent impact on human health. Although data indicates that coffee brews are capable of delivering different levels of CGAs (26.1-295.6 mg/100 mL, Tfouni et al., 2014), there is limited information regarding the influence of brewing procedures on precise levels of CGAs, delivered per cup, especially through new brewing techniques like capsules and pod. Considering the significant consumption of coffee beverages among European countries especially Portuguese consumer and due to the contribution of CGAs to human health, a comprehensive study was performed to evaluate the effect of different brewing techniques on CGAs content of coffee brews, mainly: 3-CQA, 5-CQA and 4-CQA, prepared by recent technologies. This would allow us to estimate the role of brewing techniques and the composition of coffee blends in CGAs content of coffee brews and subsequently in equilibrating the acidity of brews for consumers who suffer from acid reflux symptoms. This equilibration lets consumers avoid the consequences of high CGAs consumption and at the same time they intake sufficient amount for medicinal purposes. Table 4 and 5, summarized the literature review in terms of extraction and method of analysis along with the concentration of different CGAs derivatives in coffee beans and brews, respectively.

2.1 Chlorogenic acid content in coffee beans

Of all plants constituents, coffee has one of the highest concentrations of chlorogenic acids ranging responsible for 4-12% of the dry matter in green beans (Farah et al., 2006). There are many data available regarding the level of CGAs in coffee. Green coffee beans present high levels of CQAs, however, many procedures before brew preparation may affect levels of CGAs delivered per cup. The chemical composition of final coffee brew may be influenced by several processing steps in coffee production such as bean fermentation, bean roasting, freeze or spray

drying (in the case of instant coffee), decaffeination and/or blending (with non-coffee components) (Mills et al., 2013).

Trugo & Macrae (1984) used an extraction method with methanol and Carrez solutions I and II to determination of chlorogenic acids. Several improvements such as unambiguous peak assignment, greater chromatographic resolution and substantiation of the methods in recovery and precision were done. Trugo & Macrae, (1984) concluded that the major source of error is in the preceding extraction and clean-up procedures and not in chromatographic methods, which means there must be a strict choice of the method of extraction to reduce errors. The major problem in the chromatographic resolution was between the peaks due to 4-CQA and 3-FQA, therefore, a careful selection of the gradient and solvent conditions was found necessary. The major isomer obtained in all the samples was 5-CQA, accounting for 30% of the total CGAs, whereas the sum of CQA accounts for 70%. Trugo & Macrae (1984) obtained the highest total level of about 10.7% for a mild coffee and the lowest was 3.6% for decaffeinated coffee, suggesting that there are considerable losses of chlorogenic acid during processing. The obtained contents were for 5-CQA, 2.12, 1.67 and 1.49 mg/g for 5-CQA, 3-CQA and 4-CQA, respectively.

The CGAs content of coffee Arabica and Robusta has been extensively reported (Moon et al., 2009; Bertrand et al., 2008; Farah et al., 2005; Monteiro and Farah, 2012) although most studies in literature refer to the effect of roasting procedures. Effect of roasting on the CGAs was studied using the procedure described by Farah et al. (2005) based on the extraction of CGAs from coffee beans in 40% aqueous methanol followed by purification with Carrez reagents I and II (Trugo and Macrae, 1984) and subsequent analysis in HPLC-UV. Detection limit of 5-CQA was 0.03 µg/mL. Among total CGAs, CQAs were identified as the most abundant group which represented about 76-80% of the total CGAs followed by diCQA ranging from 15 to18% and FQA, 5.2% to 6.2%, in Arabica and Robusta coffee, respectively. A significant effect of the roasting was observed for all measured compounds. Interestingly, 3-CQA, 4-CQA and FQA exhibited higher content after 5 min of roasting. The same behaviour was observed later by Moon et al. (2009). It could be due to the isomerization of CGAs at the beginning of the roasting or partial hydrolysis of diCQAs to monoester derivatives. Dehydration of the quinic acid moiety and formation of a lactone ring yield loss of total CGAs during the longer roasting time. In all analysed samples, 5-CQA was found as the major CQA isomer (36.0-31.2 mg/g in green Arabica coffee to 42.4 mg/g in green Robusta). The decreasing order of CGAs of green beans of Arabica was 5-CQA > 4-CQA > 3-CQA > 4,5-diCQA > 3,5-diCQA > 3,4-diCQA > 5-FQA > 4-FQA > 3-FQA while for green Robusta the decreasing order was as follows: 5-CQA > 3-CQA > 4-CQA > 4,5-diCQA > 3,4-diCQA > 3,5-diCQA > 5-FQA > 4-FQA > 3-FQA (Farah et al., 2005).

Later, Farah et al. (2006) investigated commercial coffee Arabica with the purpose of investigation of decaffeination and roasting procedures on CGAs and CGLs contents of Arabica coffee. All samples were extracted in an aqueous solution of 40% methanol and purified according to the method described in detail by Farah et al. (2005) resulted in detection limit of 0.03 µg/mL. The obtained data allowed the author to confirm the 16% elevation in average CGAs levels of decaffeinated green coffee compared to regular samples. Regular samples contained

total CGAs ranging from 5.1 to 5.6% while after decaffeination, these values increase to 6.1 to 6.4%. However, 43, 42 and 35% loss in 5-CQA, 5-FQA, and 3,5-diCQA during the decaffeination process was observed. This increase in the level of other compounds was probably consequence of lixiviation of other water-soluble compounds such as carbohydrates or isomerization of the cinammoyl substituent. Regarding roasted beans, the distribution of CGA isomers between regular and decaffeinated roasted coffees was almost similar with 3-9% lower CGAs average contents in decaffeinated, compared to regular coffees probably due to changes during the decaffeination process.

Due to the higher recovery obtained from samples extracted with mixture of methanol/water, this procedure was used by Moon et al. (2009) for quantification of the level of chlorogenic acids in coffee beans. According to Moon et al. (2009) green beans presented 5-CQA ranging from 30.0 mg/g to 24.2 mg/g followed by 4-CQA (4.3-2.9 mg/g) and 3-CQA (3.2-1.7 mg/g). The general decreasing order of CQA isomers was 5-CQA > 4-CQA > 3-CQA > 3,5-diCQA > 4,5-diCQA > 5-FQA > 3,4-diCQA > 4-FQA. Regarding roasted beans, the amount of CQAs reduced significantly in accordance with the intensity of conditions ranging from 45% up to 99% for light roast (230 ºC, 12 min) and French roast (250 ºC, 17 min), respectively. The same pattern was observed by Farah et al. (2006). Isomerization of CQAs followed by formation of lactones are accounted as a reason for CQAs reduction during the roasting (Moon et al., 2009).

In case of coffee beans from different origins, 3-CQA and 4-CQA contents were increased during light roast treatment, suggesting that some CQA increase by heat treatment. As it was mentioned before, probably, high temperature of the roasting process causes a breakage of the carbon-carbon bonds of CGA, resulting in isomerization and degradation (Farah et al., 2005) before the formation of CGL and resulted in reduction of some CQA derivative like 5-CQA while causing elevation in other derivative such as 3 and 4-CQAs. Since roasting conditions play an important role in the CQA content of the coffee product, probably CQA may be used for estimation of roasting degree and light or dark roasted beans may be distinguished.

As it was seen by Farah et al. (2006) and tested then by Moon et al. (2009), 5-CQA was the predominant CGAs in all of the analysed samples (Table 4). It is worth mentioning that there are different analytical methods for measurement of CGAs in coffee, with distinct performance. Besides that, raw material (green or roasted beans) can be accounted as a main factor in the diversity of CGAs content as an increase in the levels of phenolic acids in plants has been observed in severe weather conditions such as cold, high visible light, and water stress conditions (Farah et al., 2006). Additionally, agricultural practices, climate and soil composition may also contribute to changes in CGAs content and distribution (Camacho-Cristóbal et al., 2002). All of these parameters may pose difficulties in comparing results of the presence of CGAs in coffee products.

A greater number of major and minor CGAs in green and roasted economically relevant Brazilian Arabica and Robusta coffee were investigated by Perrone et al. (2008). All samples were extracted according to the method recently described by Farah et al. (2005) and were analyzed with a LC–MS mass spectrometer fitted with an electrospray ion source. The CQAs were the most abundant CGAs class (84% and 76% of the total CGA in green Arabica and Robusta, respectively), followed by diCQAs (11% and 15% of total CGA in green Arabica and Robusta, respectively) and FQA which was responsible for 4% of CGAs in Arabica and 7% of CGAs in Robusta green beans. Similar pattern was reported previously by Farah et al. (2005). Even though green coffee has been a main source of CGAs (5–12 g/100 g) (Farah & Donangelo., 2006), data indicated that light roasting process yield an elevation of some CGAs like 3-CQA and 4-CQA which has been already reported (Farah et al., 2005; Moon et al., 2009)

Compositions of CGAs in coffee beans are extensively influenced by different steps prior to extraction like post harvesting methods such as drying. Published paper indicated that coffees processed by wet method showed higher contents of chlorogenic acids (Leloup et al., 2004; Duarte et al., 2010). In study performed by Duarte et al. (2010) coffee seeds were extracted and clarified according to Trugo and Macrae (1984) which was also described in Farah et al. (2005). The average contents of CQAs, diCQAs and FQAs in coffees treated by wet method represented 81%, 13% and 5% of total CGAs content. 5-CQA is found in the greatest amount ranging from 48.4 to 28.5 mg/g followed by 4-CQA (7.3-5.0 mg/g) and 3-CQA (5.4-3.8 mg/g). The samples treated by the semi-dry method exhibited similar distribution profile of CGAs classes to those treated by the wet method (81, 14 and 5% of total CGAs for CQAs, diCQAs and FQAs, respectively) but with regards to an average content (mg/g), coffee processed by wet method presented significantly higher total CGAs content than those processed by semi-dry method. Higher CGAs content in samples treated by wet method may contribute to water-soluble components removal during lixiviation and fermentation in the wet method or degradation of CGAs under sun heat during semi-dry method.

A potential alternative to conventional solvent extraction for the isolation of CGAs was proposed by Upadhyay et al. (2012). The ideal working range seems to be 50 ºC, with heating time of about 5 min. The experiments were then optimised for microwave power and 800 W considered as the best condition. Different solvents (ethanol, methanol and water) were tested to extract chlorogenic acids from green coffee beans when the optimal microwave-assisted extraction conditions were, 5 min, 800 W and 50 ºC. The best extraction efficiency was obtained from aqueous extract (8.9-9.0%) when compared to methanol extract (4.9-5.6%) probably due to the higher dielectric constant and polarity of water than the alcohol. Thus, better absorption of microwaves cause higher inside temperature and subsequent cell rupture occur, which results in realising compounds to the surrounding solvent. By comparing the microwave-assisted extraction and conventional extraction, significantly higher extraction yields for CGAs was achieved under optimal extraction conditions of microwave techniques which resulted in yield of 8.4% for respective method than 3.9% for conventional approach. As other techniques, it also has its drawbacks. Usually, the extraction temperature and microwave energy should be set at optimum

conditions, otherwise higher or lower temperature and power may lead to degradation of thermolabile compounds or insufficient extraction.

A simple extraction methodology based on soaking in boiled water was examined by Ayelign and Sabally, (2013) to determine the content of chlorogenic acids in coffee beans. The total content of CGAs varied from 0.9 to 46.1 mg/g in Arabica coffee samples subjected to different degrees of roasting. Similar behaviour was observed from green beans to roasted samples as have been frequently reported by other authors (Moon et al., 2009; Farah et al., 2005). The total CGAs present in coffee beans were reduced in accordance with the intensity of roasting conditions.

Generally speaking, much research has been undertaken and indicated the highly presence of CGAs, mainly: 3-CQA, 4-CQA and 5-CQA both in green and roasted Arabica and Robusta coffee. Although, the main factor that could considered responsible for drastically CGAs reduction is the roasting process however as it can be clearly seen in Table 4, roasted been, in particular light roasted beans are capable of delivering significant level of CGA.

Therefore, the further impact of using different coffee species or a blend of beans with different roasting degree on the final concentration of these compounds delivered per cup should not be discarded. Since, different steps of coffee bean production prior to extraction greatly influenced the CGAs content, this is an important issue for further comparison of brews prepared with different raw materials. Indeed, it simplifies to understand the reason of strange behaviour of the presence of CGAs prepared with the same methods but different blend or roasting intensity.

Table 4 - Overview on extraction and analytical methods used for determinations of CGAs along their content in coffee bean samples

Reference	*Sample Type*	*Extraction and Purification*	*Quantification*	*Detected Compounds*	*Concentration**
Ayelign and Sabally, (2013)	Coffee bean	2 g of ground coffee was dissolved in 100 mL of distilled water then were boiled for 5 min and filtered with 0.45 µm filter paper	HPLC – DAD ODS-C-18, 250 x 4.6 mm column Eluent A: water and acetic acid; Eluent B: methanol, Injected volume: 20 µL, Isocratic elution, Flow rate: 1 mL/min during 20 min Detection wavelength: 278 nm	Chlorogenic acid**	Total CGA: 0.9-46.1 mg/g
Monteiro & Farah, (2012)	Coffee bean	CGA were extracted with aqueous methanol (40%) and clarified with Carrez solutions (I and II).	HPLC – UV ODS-C-18, 250 x 4.6 mm column Eluent A: 80% 10 mM citric acid solution, acidity adjusted to pH 2.5 with 6 N hydrochloric acid and 20% methanol; Eluent B: methanol, Gradient elution, Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	3-CQA (4.68-5.77 mg/g) 4-CQA (6.53-7.88 mg/g) 5-CQA (35.93-39.13 mg/g)
Upadhyay et al. (2012)	Green coffee beans	In MAE ground coffee was defatted with hexane (1:6; w/v). Defatted coffee powder was extracted by means of microwave, with various solvents, such as water, methanol and ethanol. Extracts were treated with lead acetate. In conventional extraction ground coffee was defatted and extracted with water and was filtered to get a clear extract. Extracts were treated with lead acetate	UV spectrophotometer at 325 nm	Chlorogenic acid**	MAE extraction yeild: 8.4% (CGAs content: 31-62%) Conventional extraction yeild: 3.95%

Table 4- Overview on extraction and analytical methods used for determinations of CGAs along their content in coffee bean samples (cont.)

Reference	*Sample Type*	*Extraction and Purification*	*Quantification*	*Detected Compounds*	*Concentration**
Duarte et al. (2010)	Natural and parchment coffee	CGA were extracted with aqueous methanol (40%) and clarified with Carrez solutions I and II	HPLC – UV ODS-C18, 250 x 4.6 mm column Eluent A: 80% 10 mM citric acid solution, acidity adjusted to pH 2.5 with 6 N hydrochloric acid and 20% methanol; Eluent B: methanol Injected volume: 100 µl, Gradient elution, Flow rate: 1 mL/min during 60 min Detection wavelength: 325 nm (Farah et al., 2005)	3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA,5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	3-CQA (5.4- 3.8 mg/g) 4-CQA (7.3-5.0 mg/g) 5-CQA (48.4-28.5 mg/g)
Moon et al. (2009)	Green and Roasted coffee beans at different degrees	1.0 g of ground coffee beans were soaked in a methanol/water (7:3, v/v) solution and it was further treated with 0.1 mL of each Carrez solution (I and II) and 0.8 mL of methanol	HPLC – DAD C18, 250 x 4.6 mm column Eluent A: water containing 0.1% of formic acid, Eluent B: acetonitrile containing 0.1% of formic acid, Injected volume: 10 µL, Gradient elution, Flow rate: 0.8 mL/min during 57 min Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Green beans: 3-CQA (1.7-3.2 mg/g) 4-CQA (4.3-2.9 mg/g) 5-CQA (30.2-24.2 mg/g); Roasted beans: 3-CQA (0.5-1.6 mg/g) 4-CQA (0.7-1.8 mg/g) 5-CQA (1.1-3.0 mg/g)
Bertrand et al. (2008)	Coffee bean	250 mg of dried powder coffee was placed with 80 mL of aqueous methanol (70% w/w) and 1 mL of aqueous acetic acid (50:50 v/v)	HPLC – UV Uptisphere ODB, 250 x 4.6 mm column Eluent A: methanol, Eluent B: 2 mM phosphoric acid, Gradient elution, Flow rate: 1 mL/min during 50 min, Detection wavelength: 327 nm	3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, CFQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, CA, CTR, CT	CQAs content in coffees from different location and genotype: 3-CQA (3.29-4.45 mg/g) 4-CQA (5.09-6.21 mg/g) 5-CQA (37.61-46.51 mg/g)
Perrone et al. (2008)	Green and Roasted Coffee beans	Half a gram of ground coffee was suspended in 60 mL of 40% aqueous methanol and 1.0 mL of each Carrez solution (I and II) were added for purification	HPLC – DAD Magic C30, 150 x 2 mm column Eluent A: 0.3% aqueous formic acid, Eluent B: methanol, Gradient elution, Flow rate: 0.2 mL/min during 100 min, Detection wavelength: 370 nm	3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3-*p*-CoQA, 4-*p*-CoQA, 5-*p*-CoQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA, 3,4-diFQA, CFQA	Green bean: 3-CQA (6.67-10.65 mg/g) 4-CGA (7.709-12.77 mg/g) 5-CQA (33.57-41.14 mg/g) Roasted beans (very light to very dark): 3-CQA (0.51-13.08 mg/g) 4-CGA (0.55-16.66 mg/g) 5-CQA (0.93-31.75 mg/g)

Table 4 - Overview on extraction and analytical methods used for determinations of CGAs along their content in coffee bean samples (cont.)

Reference	*Sample Type*	*Extraction and Purification*	*Quantification*	*Detected Compounds*	*Concentration**
Farah et al. (2006)	Green and Roasted coffee beans (Regular and decaffeinated)	Half a gram of ground coffee was suspended in 60 mL of 40% aqueous methanol and clarified with 1.0 mL of each Carrez solution (I and II)	HPLC – UV ODS-C18, 250 x 4.6 mm column Eluent A: 80% 10 mM citric acid solution, acidity adjusted to pH 2.5 with 6 N hydrochloric acid and 20% methanol, Eluent B: methanol, Gradient elution, Flow rate: 1 mL/min during 60 min, Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Regular green beans: 3-CQA (3.4-5.4 mg/g) 4-CQA (5.2-6.4 mg/g) 5-CQA (30.6-43.4 mg/g) Decaffeinated green beans: 3-CQA (16.7-18.8 mg/g) 4-CQA (15.7-19.9 mg/g) 5-CQA (19.3-22.6 mg/g)
Farah et al. (2005)	Green and Roasted coffee beans at different degrees	Half a gram of ground coffee was suspended in 60 mL of 40% aqueous methanol and 1.0 mL of each Carrez solution (I and II) were added for purification	HPLC – UV ODS-C-18, 250 x 4.6 mm column Eluent A: 80% 10 mM citric acid solution, acidity adjusted to pH 2.5 with 6 N hydrochloric acid and 20% methanol, Eluent B: methanol, Gradient elution, Flow rate: 1 mL/min during 60 min, Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 3-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Green bean: 3-CQA (4.7-9.2 mg/g) 4-CGA (5.4-6.0 mg/g) 5-CQA (31.2-42.6 mg/g) Roasted beans (very light to very dark): 3-CQA (0.5-12.5 mg/g) 4-CGA (0.5-14.8 mg/g) 5-CQA (1.0-38.0 mg/g)
Trugo & Macrae, (1984)	Instant coffee powder	Ground coffee dissolved in 80 mL of an aqueous methanol solution with the addition of 2 mL of Carrez solution I and II	HPLC – DAD Spherisorb 5-ODS column Eluent A: methanol, Eluent B: 0.01 M tripotassium citrate solution (pH 2.5), Injected volume: 100 µL, Gradient elution, Flow rate: 1 mL/min during 60 min, Detection wavelength: 325 nm	3-CQA; 4-CQA; 5-CQA; 3-FQA; 4-FQA; 5-FQA; 3,4-DiCQA; 3,5-DiCQA and 4,5-DiCQA	Coffee powder: 3-CQA (0.70-1.89 mg/g) 4-CGA (0.81-1.70 mg/g) 5-CQA (1.02-3.50 mg/g)

* In some references, data were reported as mg/100g. In the respective table data were exhibited as mg/g in order to facilitate the comparison.

** The common name of "chlorogenic acid" was mentioned in published paper. Although "chlorogenic acid" is the alternative name for 3-CQA (PubChem, 2013), it should be used cautiously so the use of this name in table was avoided.

2.2 Chlorogenic acid content in coffee brews

The effect of brewing modes on chemical composition of final beverage is frequently reported by authors (Ratanayak et al., 1993; Bell et al., 1997; Ludwig et al., 2012; Tfouni et al., 2014; Mills et al., 2013). With regards to CGAs, most studies found in literature focus on the determination of CGAs in classical coffee brewing techniques like boiled, filter and instant coffee. As mentioned before, coffee is the main source of CGAs. Regardless of different chemical composition of Arabica and Robusta beans and primary processing methods used, brewing techniques seems to be another important procedure which cause differences in CGAs delivered per cup of coffee prepared from the same roasted and ground coffee but different preparation methods. So the preparation techniques have received special attention due to their effect on CGAs of coffee brews. Therefore, it became essential to study the concentrations and distributions of these compounds in coffee brews in order to evaluate exposure to precise levels delivered per cup of coffee.

In order to study the effect of brewing procedure (boiled and filter), Tfouni et al. (2014) used a clean-up method described by Trugo & Macrae, (1984) and the analysis was carried out using HPLC-DAD at 324 nm to quantify CQA isomers including 3-CQA, 4-CQA and 5-CQA. The sum of CQAs in brews ranged from 24.2 to 295.6 mg/100 mL for Arabica and from 30.4 to 253.8 mg/100 mL for Robusta samples, although the differences were found insignificant. In brews prepared with Arabica coffees, 5-CQA was assumed to be 33-43% of total CQAs and 34-41% for beverages brewed from Robusta coffees. In both coffee species and preparation modes, the highest concentration of CQAs corresponds to brews obtained from light roasted degree and consequently the lowest concentration corresponds to samples extracted from dark roasted beans. The results obtained by Tfouni et al. (2014) are in accordance with the ones that have been reported by other authors (Crozier et al., 2012; Hečimović et al., 2011; Jaiswal et al., 2013), where brews prepared from darker roasted coffees present lower CQAs content. Comparing two brewing procedure, filter coffees are the ones that would least contribute to CQAs intake while boiled coffee showed higher content of CQAs at the same roasting degree. This can be explained by the fact that during boiled coffee procedure, roast and ground coffee stays in contact with water for a longer period, increasing the extraction yield (Tfouni et al., 2014).

Regarding the espresso coffee, Caprioli et al. (2013) quantified the CGAs (3-CQA, 5-CQA, 3,5-diCQA) in espresso coffee prepared with different extraction time with two different blends using two different coffee machines. For this purpose, they purified brewed coffee using SPE and analysed the clean extract by means of LC-DAD (Table 5). Caprioli et al. (2013) indicated that the type of coffee machine may influence the final concentration of CGAs in espresso coffee due to different applied temperature during the percolation time. CGAs concentration was found higher in Arabica than Robusta coffee. In this study CGA isomers concentrations were in decreasing order 5-CQA > 3-CQA > 3,5-DiCQA as observed in previous studies (Tfouni et al., 2014; Trugo & Macrae, 1984). Considering the total concentration of CGAs in respective espresso coffees, Arabica coffee presented slightly higher concentration (1733.7-2223.5 mg/L) in

comparison to Robusta (1522.5-2122.5 mg/L), although differences were not significant. These results are in agreement with Tfouni et al. (2014) who found almost same concentration for CQAs in brewed coffee prepared from Arabica and Robusta. Among studied compounds, 5-CQA was identified as the main CGAs with higher concentration (986.1-1559.9 mg/L) which was in agreement with findings previously reported who found 5-CQA as the main CGAs isomer (Tfouni et al., 2013; Trugo & Macrae, 1984). They proved that CGAs were mainly extracted at the beginning of brewing procedure (first 10 s).

The most abundant phenolic compounds in all analysed coffee samples prepared from commercial coffee blends by Mills et al. (2013) were CQAs. They clarified coffee brews with Carrez reagents (I and II) and samples were analysed using LC-MS for identification of main CGAs in the extract followed by analysis with HPLC for quantification of individual compounds. The predominant CQA were 5-CQA accounting for between 25-30% of the total CGAs (8.1-41.0 mg/200 mL). The subsequent phenolic compounds were detected in decreasing order as follows: 4-CQA (4.1-17.1 mg/200 mL) > 3-CQA (3.6-16.2 mg/200 mL) > 5-FQA > 4-FQA such as the previous studies (Caprioli et al., 2013; Tfouni et al., 2014; Trugo & Macrae, 1984). The CGAs content of the analysed coffee samples ranged from 27.3 to 121.2 mg/200 mL coffee, demonstrating that coffee selection (different variety or commercial coffee contain other ingredients) may have a profound influence on an individual's intake of CGAs as less CGAs concentration was obtained in brews prepared from French roasted coffee (high roasted intensity). Great CGAs content was related to Nescafé green which contain a proportion of green and unroasted coffee so less CGAs were lost during roasting due to degradation or Maillard reaction. Although the instant coffee that they assessed passed through several production steps, they were capable of delivering similar amounts of CGA per serving like commercial fresh ground coffee (Mills et al., 2013)

Filter and soluble coffee (regular and decaffeinated) were tested in terms of CGAs derivatives by Rodrigues & Bragagnolo, (2013). Around 37 mL of Filter coffee and 10 mL of soluble coffee were prepared in volume of around 38 and 10 mL, respectively. 5 mL of each extract was dried and reconstitute in 1 mL of water and purified. Determinations of CGAs were performed based on mg/100 g dry brew extract. The respective method was validated through LOD (2.0 μg/mL), LOQ (6.1 μg/mL), repeatability (2.5%) and recovery (101-104%) of 5-CQA. Isomers of CQA were considered as the most abundant class of CGAs accounting for 1495.0 and 4910.3 mg/100 g dry extract of coffee brew which was the greatest amount of CGAs observed such as other studies (Caprioli et al., 2013; Mills et al., 2013; Tfouni et al., 2014; Trugo & Macrae, 1984). However, regular roasted and ground filter coffee brews showed about 2–4 times higher total CGAs than regular soluble coffee.

Regarding CGAs content in coffees which mainly consumed by consumers, Crozier et al. (2012) studied different espresso coffee provided from different outlets along with brewed

coffee from pure Arabica with different roasting intensity in order to investigate the possible reason for variation in the CGAs contents of coffees. The less obtained quantity of CQAs was 24 mg/27 mL, where 21% corresponds to 3-CQA, 29% to 4-CQA and 50% to 5-CQA, while greatest achieved concentration was 422 mg/52 mL where 23% corresponds to 3-CQA, 26% to 4-CQA and 51% to 5-CQA. These obtained results are in accordance with the ones that have been frequently reported by other authors (Caprioli et al., 2013; Mills et al., 2013; Tfouni et al., 2014; Trugo & Macrae, 1984). A great variability was found in the CGAs content of the analysed samples (24-422 mg per cups varied from 23-100 mL). The large variability in CQAs content could be due to a number of factors like, batch-to-batch differences in the Arabica beans, roasting procedures, grinding conditions and as well as the coffee-making process (temperature of water/steam in the extraction vessel and its duration). Crozier et al. (2012) also observed a bigger loss of CGAs during roasting process that resulted in 3-CQA and 4-CQA being destroyed more slowly than 5-CQA.

The composition of coffee brew and the level of micronutrient contents of coffee brews may be influenced by variety of coffee beans, blending, roasting degree, grinding and subsequent brewing methods. Concerning brewing modes, times of percolation play and important role as extracting compounds significantly alter along with time. For this purpose, Ludwig et al. (2012) studied the influence of brewing time on CQAs in two different brews. As it has been extensively reported (Caprioli et al., 2013; Mills et al., 2013; Tfouni et al., 2014; Trugo & Macrae, 1984), 5-CQA was found as the most important compound among other CQAs in all analysed samples, followed by 4-CQA and 3-CQA. In contrast to other published papers (Farah et al., 2005; Moon et al., 2009) espresso coffees with Arabica presented higher CQAs than those in Robusta, probably due to different origins or greater CGAs reduction in Robusta coffee during roasting (Clifford, 1997; Perrone et al., 2010). Determination of CQAs in time portions of espresso coffee was in agreement with those previously reported (Caprioli et al., 2013) and exhibited the more than 70% antioxidant extraction during the first 8 s while in filter coffee higher extraction was obtained at the end of percolation time. When comparing brewing methods, espresso coffee showed higher content of 5-CQA (52.9-96.7 mg/100 mL), 4-CQA (35.0-55.6 mg/100 mL) and 3-CQA (25.8-43.2 mg/100 mL) with respect to filter coffee (21.8-38.7, 19.4-25.3, 15.0-17.0 mg/100 mL for 5-CQA, 4-CQA and 3-CQA, respectively). This may be due to the technological differences between espresso brewing technique and filter coffeemaker. The high water pressure applied in espresso coffeemaker favours the extraction process. This could be also explained by the lower volume of espresso (46-47 mL) than filter coffee (520-532 mL).

In the case of Fujioka & Shibamoto, (2008), results were in accordance with the previous studies (Caprioli et al., 2013; Ludwig et al., 2012; Mills et al., 2013; Tfouni et al., 2014; Trugo & Macrae, 1984) where 5-CQA was the most important CQAs in analysed samples. Ground-roasted coffee (12.5 g) was brewed with 450 mL of water to prepare filter coffee. Brewed coffees were cleaned-up using a mixture of Carrez solutions and methanol and data were reported as mg/g of ground coffee. The total CQAs ranged from 5.2 to 17.1 mg/g in regular coffees and from 2.1 to 16.1 mg/g in decaffeinated coffees. 5-CQA was presented at the highest level (2.1- 7.0 mg/g of

coffee), and comprising 36-42% and 37-39% of the total CGAs in regular and decaffeinated coffees, respectively.

CGA standards have been synthesised in many steps and many standards being practically unfeasible or costly. These difficulties have led to a restricted commercial availability of these compounds. As it has been extensively discussed, the prominent CGAs are related to class of CQAs, mainly 3-CQA, 4-CQA and 5-CQA. Since we have some limitations in expenses, these main three standards were chosen as the representative of CGAs in coffee brews. Different methodologies may be used for the extraction of CGAs from coffee brews. The method of clarification of brews with Carrez reagents according to Trugo & Macrae, (1984) was extensively used by author and was selected for CQAs extraction from coffee brews. This method was adopted and validated for coffee brews by Fujioka & Shibamoto, (2008). Lower sample volume and reagents make this method more environmental friendly and let us to prepare more samples in short time when compared to Tfouni et al. (2013) method. Therefore, this method was selected for comprehensive study of these bioactive compounds in various types of coffee brews. As already referred, authors described the presence of CGAs in coffee beverage, although the information given about their contents in various types of coffee brews was rare. Among the several brewing techniques, special attention was given to filter, boiled and instant coffee. As far as authors knowledge, there was no or limited information about simple and popular brewing procedures such as coffee pod and capsules which are accepted among Portuguese consumers, or other methods of extractions like mocha and French press.

So in the present study, concentrations of the main isomers of CQAs (3-,4- and 5-CQA) were evaluated in a wide range of coffee brews which are commonly used by consumers especially by Portuguese people.

Table 5 - Overview on extraction and analytical methods used for determinations of CGAs along with their content in various coffee brews

Reference	*Sample Type*	*Purification Technique*	*Quantification*	*Detected Compounds*	*Concentration**
Tfouni et al. (2013)	Coffee brew (Filtered and Boiled)	5 mL of coffee brews were diluted in methanol/water 20:80, v/v) and purified with 2mL of each Carrez reagents I and II	HPLC – DAD Lichrosphere-C18, 250 x 4 mm column Eluent A: Acetonitrile, Eluent B: Water adjusted with phosphoric acid until pH 2.7, Injected volume: 20 µL, Gradient elution, Flow rate: 1 mL/min during 45 min, Detection wavelength: 324 nm	3-CQA, 4-CQA, 5-CQA	Arabica coffee (light to dark roasted degree), Total CQAs: 24.2-219.1 mg/100 mL (Filter coffee) 26.1-295.6 mg/100 mL (boiled coffee) Robusta coffee (light to dark roasted degree), Total CQAs: 30.4-187.7 mg/100 mL (Filter coffee) 41.3-253.8 mg/100 mL (boiled coffee)
Caprioli et al. (2013)	Coffee Brew (Espresso)	0.5 mL of brew diluted in with 2 mL of water was purified with SPE Strata-X extraction cartridge conditioned with methanol (3 mL) followed by water (6 mL). The solution was filtered and analysed.	LC – DAD Polar-RP 80Å, 150 x 4.6 mm column Eluent A: water and 0.1% of formic acid, Eluent B: methanol and 0.1% of formic acid, Injected volume: 5 µL, Gradient elution, Flow rate: 1 mL/min during 18 min, Detection wavelength: 325 nm for 5-CQA and 330 nm for 3-CQA	3-CQA, 5-CQA, and 3,5-diCQA	Total CGAs: 222.3-173.3 mg/100 mL (Arabica); 212.2-152.2 mg/100 mL (Robusta)
Mills et al. (2013)	Coffee brew (Instant)	Clarification of 4 mL of coffee brew with 1.0 mL of each Carrez solutions (I and II), along with 0.8 mL ethanol.	HPLC – PDA C18 (250 x 4.6 mm) column Eluent A: 5 N hydrochloric acid (0.1%) in 95% water and 5% methanol, Eluent B: 5 N hydrochloric acid (0.1%) in 50% acetonitrile and 50% water, Injected volume: 50 µL, Gradient elution, Flow rate: 0.7 mL/min during 60 min, Detection wavelength: 320 nm	3-CQA, 4-CQA, 5-CQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Commercial fresh ground: Total CGAs (27.3-94.4 mg/200 mL) Commercial instant coffee: Total CGAs (37.0-121.2 mg/200 mL)
Rodrigues & Bragagnolo, (2013)	Coffee brew (Roasted ground coffee and soluble coffee)	Dried coffee brew extract were dissolved in 1 mL of water and teated with o.1 mL of each Carrez solutions (I and II) along with 0.8 mL of methanol	HPLC – DAD ODS-C18, 250 x 4.6 mm column Eluent A: 80% 10 mM citric acid solution (pH 2.5) and 20% of methanol Eluent B: 100% methanol Injected volume: 5 µL, Gradient elution, Flow rate: 1 mL/min during 70 min Detection wavelength: 325 nm	CQAs, FQAs, diCQAs, CoCQAs, Other derivative (3F,4CQA, 3C,4FQA, 3C,5FQA, 4F,5CQA, 4C,5FQA)	Total CQA: Regular roasted ground coffee: 2138.8-4910.3 mg/100 g dried extract Regular soluble coffee: 1495.0-1870.3 mg/ 100 g dried extract

Table 5 - Overview on extraction and analytical methods used for determinations of CGAs along with their content in various coffee brews

Reference	*Sample Type*	*Purification Technique*	*Quantification*	*Detected Compounds*	*Concentration**
Crozier et al. (2012)	Coffee brew (Espresso)	Coffee brew was diluted with methanol (50-fold) and directly injected for analysis.	HPLC – PDA Polar-RP 80Å, 250 x 4.6 mm column Mobile phase: Gradient elution of 5-25% acetonitrile containing 1% formic acid, Injected volume: 5 µL, Flow rate: 1 mL/min during 60 min, Detection wavelength: 325 nm (Adopted from Stalmach et al., (2006)	3-CQA, 4-CQA, and 5-CQA	Total CGA: Ranging from 24-422 mg per cup of 23-100 mL
Ludwig et al. (2012)	Coffee brew (Espresso and Filter)	Samples were treated with 5 mL of methanol and 3 mL of distilled water. It was used 20 mL of methanol/water mixture (70:30)	HPLC – DAD Hypersil-ODS, 250 x 4.6 mm column Eluent A: methanol, Eluent B: Water acidulated with phosphoric acid (pH 3.0), Injected volume: 100 µL, Gradient elution, Flow rate: 0.8 mL/min during 27 min, Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Espresso coffee: 3-CQA (25.8-43.2 mg/100 mL) 4-CQA (35.0-55.6 mg/100 mL) 5-CQA (52.9-96.7 mg/100 mL) Filter coffee: 3-CQA (15.0-17.0 mg/100 mL) 4-CQA (19.4-25.3 mg/100 mL) 5-CQA (21.8-38.7 mg/100 mL)
Fujioka & Shibamoto, (2008)	Coffee brew (Regular and decaffeinated coffee)	Samples (3 mL) were treated with 0.1 mL of each Carrez solutions (I and II) and 0.8 ml of methanol	HPLC – DAD C18, 150 x 4.6 mm column Eluent A: 10 mM citric acid solution, Eluent B: methanol, Injected volume: 5 µL, Gradient elution, Flow rate: 1 mL/min during 85 min, Detection wavelength: 325 nm	3-CQA, 4-CQA, 5-CQA, 3-FQA, 4-FQA+5-FQA, 3,4-diCQA, 3,5-diCQA and 4,5-diCQA	Regular coffee: 3-CQA (1.32-3.95 mg/g) 4-CQA (1.44-4.56 mg/g) 5-CQA (2.13-7.06 mg/g) Decaffeinated coffee: 3-CQA (0.45-3.42 mg/g) 4-CQA (0.51-3.78 mg/g) 5-CQA (0.82-6.23 mg/g)

*Results of mg/L were presented as mg/100 mL to simplify the comparison of CGAs concentration in different brews.

2.3 Aim of the thesis

To the authors' best knowledge limited studies have been performed with regards to the quantification of CQAs content in wide range of coffee brews especially brews prepared by recent technologies like pod, variety of capsules or easy drinks such as iced coffee. For this purpose, in the present research, a comprehensive study was done to estimate the presence of caffeoylquinic acids namely: 3-CQA, 4-CQA and 5-CQA as the main chlorogenic acids isomers to determine the abundant CQAs isomers in coffee brews.

A wide range of coffee brews from decoction, infusion and pressure methods along with soluble coffees (instant, iced coffee and iced cappuccino) were analysed to assume the effect of brewing mechanisms in the presence of CQAs and to evaluate the amount of these compounds delivered per cup. To study the effect of coffee species, different brewed coffees were produced with pure Arabica and Robusta coffee to compare the CQAs content in each type of brew. The method validation was then accomplished, through quantification parameters like linearity, limits of detection and quantification, sensitivity and precision and accuracy.

The isomers 3-, 4- and 5-CQA, such as caffeoylquinic acids were considered in coffee prepared by many different brewing processes such as: boiled, French, mocha, filter, espresso (different types of capsule and POD), instant and by other pressure processes like from vending machines and typical coffee from a coffee bar in city. The goal of this research was to study the levels of chlorogenic acids in the principle brewing techniques of taking a coffee adapted for Portuguese people since these compounds have important healthy aspects which makes people very interested in more information about the subject.

Chapter 3

Material and Methods

3.1 Reagents and Standards

All reagents or solvents were for analytical or HPLC grade. Solvents were acetonitrile and methanol (HPLC gradient grade) and were obtained from VWR (Belgium). Citric acid was supplied from Merck (Germany). Filtered water used for HPLC analysis was prepared by vacuum purification through 0.45 μm filter membranes. Zinc acetate dihydrate (CAS: 5970-45-6) (VWR, Belgium), glacial acetic acid (Merck, Germany) and Potassium hexacyanoferrate (II) trihydrate (CAS: 14459-95-1) (VWW, Belgium) were used to prepare Carrez solutions I and II.

Referenced standard of 5-caffeoylquinic acid (Neochlorogenic acid, $C_{16}H_{18}O_9$, CAS: 906-33-2) (≥95.0% purity) were purchased from Cymit(Barcelona, Spain). Individual standard of 4-caffeoylquinic acid (4-O-caffeoylquinic acid, $C_{16}H_{18}O_9$, CAS: 905-99-7) (≥95.0% purity) and of 3-caffeoylquinic acid (chlorogenic acid, $C_{16}H_{18}O_9$, CAS: 327-97-9) (≥95.0% purity) were acquired from Sigma – Aldrich (MO, USA).

3.2 Standards preparation

Individual stock solutions of 3-, 4- and 5-CQA with concentration of 100 mg/L were prepared in aqueous methanol (10% v/v) to determine the retention time of CQAs through the column. Similarly, a stock solution containing all CQAs was prepared in aqueous solution of methanol (10% v/v) with the following concentration: 3-CQA (400 mg/L), 4-CQA and 5-CQA (200 mg/L). Calibration standards in aqueous methanol (10% v/v) were prepared for concentration levels ranging from 2 to 400 mg/L for 3-CQAs and between 1-200 mg/L for 4-CQA and 5-CQA. Standards were protected from light using amber glass vials or aluminium foil and stored at – 22 °C.

3.3 Equipment

The instrumental analysis of CQAs was performed using HPLC-DAD, Merck Hitachi Elite LaChromatograph (Tokyo, Japan) equipped with a quaternary system of pumping (L-2130) and L-2200 auto sampler with L-2455 UV/vis spectrophotometry diode array detector. Separation was achieved using LiChroCART® RP-18 end-capped (250 x 4 mm, 5 μm) column, attached to a guard

column (4 x4 mm, 5 µm) of the same kind. EZChrom Elite 3.1.6 software was used for data acquisition and peak integration.

The pH of mobile phase was controlled by a pH meter (pH Meter GLP 21, Crison, (EEC)). To proceed to the extraction it was necessary to use a centrifuge Rotofix 32A (Germany).

3.4 Samples

Roasted Arabica coffee (100% *Coffea arabica*, 2.34% water content) and roasted Robusta coffee (100% *Coffea robusta*, 3.11% water content) packaged in protective atmosphere were kindly supplied by a local company in Porto, Portugal. Samples were transported to the lab and kept at -20 ºC until analysis. Roasted beans were ground by means of a home grinder (Braun 4041, Mexico) to obtain roasted and ground coffees. Prior to extraction all ground coffee, both Arabica and Robusta coffee, were sieved to estimate the particle size distribution in coffees. In order to determine the particle size, 50 g of ground coffee were sieved by means of 3 laboratory test sieves (Retsch, Germany) in different mesh size (212, 300 and 500 µm). Then the particles of each sieve were weighted and presented as percentage from total mass. As it can be clearly seen in Figure 5, ground Arabica and Robusta coffee have almost the same particle distribution. Since particle sizes of these two species were almost the same, the effect of grinding size on extraction of target compounds was at minimum. These ground coffee was used to prepare different brews namely: boiled, French, filter and mocha.

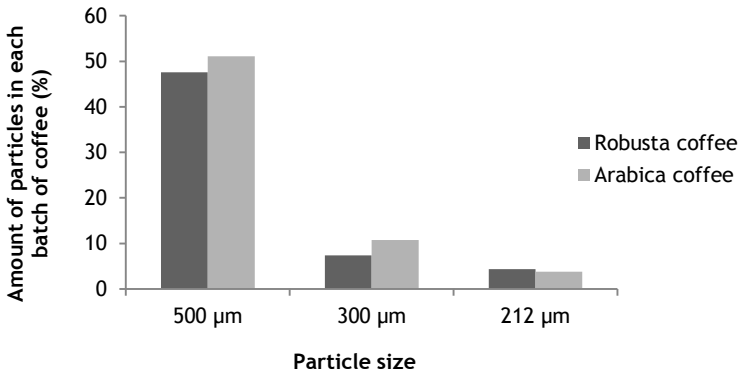


Figure 5 - Particle size distribution in roasted and ground pure Arabica and Robusta coffees.

Since espresso coffee should be prepared with fine particles, different grinds size was used (Figure 6) for espresso lab made. Coffee beans were ground before brewing (La Cimbali®, grinder-doser 6/SA) and ECs were prepared using a semiautomatic espresso machine (La Cimbali M31 Classic). In order to prepare a high quality espresso coffee, a range of particle size distribution from course to very fine ground is required. As it can be seen, grinds contained a high percentage of medium size particles (300 µm).

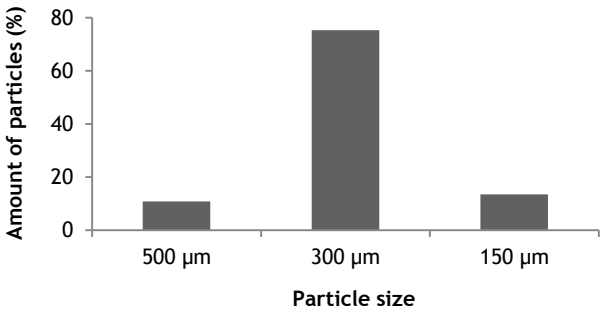


Figure 6 - Particle size distribution in roasted and ground pure Arabica coffee used for espresso preparation.

Various brands of different types of coffee were also purchased randomly from a local market and coffee bars in Porto, Portugal. Commercial coffee blend used for brewing filter coffee along with iced coffee and iced cappuccino were supplied by a local company from Colombia. A description with regards to the coffee beans used for brew preparation was exhibited in Table 6.

Table 6 - Description of commercial coffees used for preparation of various types of coffee brews*

Commercial coffee	*Description*	*Roasted condition*
Roasted and ground Arabica coffee	100% Arabica, humidity of 2.34%	NA**
Roasted and ground Robusta coffee	100% Robusta, humidity of 3.11%	NA
Commercial coffee blend	100% Colombian coffee	NA
Capsule A – Type 1	A blend of south America and east Africa Arabicas with a touch of Robusta	Roasted intensity of 10***
Capsule A – Type 2	South and Central American Arabica with Robusta	The balance of lightly roasted; Roasted intensity of 8***
Capsule A – Type 3	Blending South American Arabica with a touch of Robusta	Roasted intensity of 5***
Capsule A – Type 4	A pure and lightly roasted Arabica from South America	Roasted intensity of 4***
Capsule A – Type 5	A blend of Arabica from Latin America and Asia	Long roasting at low temperature; Roasted intensity of 11***
Capsule B	Blend of Arabica and Robusta	Medium roasted
Capsule C	100% Arabica coffee	NA
Pod espresso	Blend of Arabica and Robusta	NA
Instant natural A	Soluble coffee natural	NA
Instant natural B	NA	NA
Instant decaffeinated	Soluble coffee decaffeinated	NA
Instant espresso	100% pure Arabica from Central and South America	NA
Iced coffee	NA	NA
Iced cappuccino	NA	NA
Vending coffee	NA	NA
Coffee bars	NA	NA

* All information was adopted from the label of coffee products,
**Not available,
*** Roasted intensity was defined ranging from 1 to 12 where 1 is very light and 12 is very dark roasted intensity.

3.5 Preparation of coffee brews

The purpose of the sampling scheme was to evaluate chlorogenic acid concentration in a comprehensive range of coffee brews commonly consumed by people. A total of twenty-five coffee brews were prepared according to the manufacturers' instructions, however information about the coffee origins and species or roasting conditions used to prepare the blends was not available for commercial coffees. Coffee brews were stored at -22 ºC in polypropylene cups until duplicate analyses of the presence of 3-CQA, 4-CQA and 5-CQA with the analytical methods explained further on. Three cups of coffee were prepared for each type of brew and mixed together to obtain a homogeneous solution.

3.5.1 Regular roasted and ground Arabica and Robusta coffee brews

The process of coffee brew preparation, although fundamentally simple for the consumer, is complicated and numerous technical approaches should be considered for production of high quality coffee brew. In this part, all coffee samples, except espresso coffee which was prepared

with coffee/water ratio of 7.5/40 mL, were prepared with a coffee/water ratio of 7.5 g/100 mL, to simplify the comparison of brewing techniques in terms of CQAs content. The preparation modes were:

Scandinavian-type boiled coffee: it was prepared by boiling ground beans (11.25 g of pure Arabica or Robusta) with 150±3 mL of distilled water for 10 min followed by 2 min of settling time. The liquid was decanted. Individual cup size was 150 mL.

French press coffee: it was brewed by pouring 150±3 mL boiling water on to 11.25 g of ground coffee powder in glass French press pot followed by stirring. After 2.5 min, the coffee brew was separated from ground coffee by pressing the plunger. Individual cup size was 150±3 mL (Silva et al., 2012).

Mocha coffee: it was brewed using an aluminum mocha pot. Around 11.25 g ground coffee was placed in filter cup. Mocha pot was filled with 60±3 mL of cold water. The pot was heated until the water reservoir was empty.

Filter coffee: 22.5 g of roasted and ground coffee were put in a paper filter bag (Nº 2) and extracted with 300 mL of boiled distilled water by means of conventional percolation coffee machine KRUPS Aroma Café 5 (Germany). The brew dripped into a heated pot within 2-5 min. The individual cup size was 150 mL (Silva et al., 2012).

Espresso coffee: was prepared using 7.5 g of finely roasted and ground Arabica coffee using a semiautomatic espresso machine (La Cimbali M31 Classic) with hot water (90±2 °C, temperature of water at the exit of the heating unit) under pressure (9.0±0.2 bar) during 21±3 s until the volume in the cup met 40±3 mL (Andueza et al., 2003).

3.5.2 Commercial coffee brews

Coffee brews were prepared according to the manufacturers' instructions or obtained from local bars in Porto, Portugal.

Capsule coffee: extraction of each capsule was performed using a coffee capsule system (KRUPS, XN2100, Germany) at a pressure of 19 bar by hot water (90-95 ºC). All capsules consisted of a plastic cylinder covered by an aluminum film. The amount of coffee in each capsule was as follows: A-type 1 (6.008±.003 g), A-type 2 (5.01±0.06 g), A-type 3 (5.01±0.03 g), A-type 4 (5.14±0.02 g), A-type 5 (6.13±0.11 g), B (5.19±0.11 g), C (5.71±0.02 g). Each cup contained 40±3 mL of coffee brew.

Coffee pod: it was brewed using the SGL coffee machine, designed for pod. The size of a single serving was 40±3 mL derived from the brewing of a 7.08±0.15 g roasted and ground coffee.

Instant coffee: for this purpose, 2 g of commercial instant coffee powder was extracted with 150±3 mL of boiled water.

Iced Cappuccino: it was prepared based on preparation instructions as one pack was put in the glass and 100±3 mL of cold drinking water was added and well stirred.

Iced coffee: it was prepared based on preparation instructions where 2 table spoons of powder (8 g) were put in a glass and 240±3 mL of cold drinking water was added and stirred well.

Vending coffee: it was obtained from Necta Coffee Vending Machine (Necta Astro Double Brew) to draw a cup of coffee of about 30±3 mL. The brew was poured directly to polypropylene test tubes.

Coffee bars: they were obtained from coffee bars stores in Porto, Portugal.

3.6 Sample Extraction and clean up

To proceed to clean up, Carrez solutions I and II were used for the precipitation of proteins, elimination of turbidity and breaking of emulsions. Carrez solution I was prepared by dissolving 21.9 g of zinc acetate and 3 mL of glacial acetic acid in distilled water and diluting to 100 mL. Carrez solution II was prepared with 10.6 g of potassium hexacyanoferrate (II) in 100 mL of distilled water. The solutions were kept in refrigerator (4 ºC)

Prior to extraction, three cups of each type of coffee brew were defrosted, mixed and heated to reach a homogeneous mixture at 40-45 ℃. Extractions were done in duplicated according to the method of Fujioka & Shibamoto, (2008). To proceed to extraction, 3 mL of coffee was transferred to a polyethylene test tube and treated with 0.1 mL of each Carrez solution (I and II) and 0.8 mL of methanol. Analysis of the clean extract resulted in peak area out of the linearity range, therefore, the volume was made up with distilled water to 8 mL (diluted solution contain 10% methanol), due to high concentrations of chlorogenic acids in some coffee samples. Chlorogenic acids at very high concentrations, when diluted, will be in the linearity range. The mixture was vortexed for 1 min and left to stand 10 min. After centrifugation (4000 rpm, 10 min), the upper phase was filtered through a 0.2 µm filter (PTFE, VWR, USA) and used directly for analysis with HPLC-DAD at 325 nm. Concentrations of CQAs were calculated after applying the dilution factor of 2.5.

3.7 Chromatographic conditions

Quantitative analysis of chlorogenic acids was performed using HPLC-DAD system based in method described previously by Tfouni et al. (2014). The mobile phase composition was eluent A: 10 mM citric acid solution, acidity adjusted to pH 2.4 and eluent B: acetonitrile. The gradient was programmed as follows: from 0 to 30 min 8% of B, 30 to 35 min increase to 80% of B, 35 to 40 min 80% of B, 40 to 45 min decrease to 8% of B, 45 to 50 min 8% of B. Injected volume was 10 µL and the flow rate of analysis was 1 mL/min. Detection of CQAs was carried out at 325 nm. Identification of the analytes was confirmed by retention time and spectrum comparison with standard solutions.

3.8 Quality assurance and control

Chlorogenic acids are the most important phenolic compounds present in coffee. Individual standards and stock solution should be preserved in freezer (-22 ºC) to protect standards from isomerization. To avoid the isomerization of CQAs during the storage, all extracts were prepared and analysed freshly. Besides that, blanks were extracted and analysed in order to identify contamination. It wasn't found any contamination during the analysis of blanks.

Since separation of CQAs is pH dependent, pH of mobile phase has to be measured before analysis, therefore mobile phase was prepared freshly and the pH was checked to be 2.4.

3.9 Waste treatment

The waste generated in this work consisted of organic solutions containing acetonitrile and traces of chlorogenic acids and citric acid. Besides that, a mixture of coffee, carrez solutions and methanol was obtained during extraction method. All these residues were collected in closed containers, properly labelled for further treatment by the Environmental Management System of FEUP – EcoFEUP.

3.10 Statistical Analysis

To evaluate differences in variation between Arabica and Robusta, a one-way ANOVA was performed with a level of significance of 95%. Data are reported as mean ± standard deviation of two extraction followed by two injection. All statistical analysis was carried out by Minitab 17 software. Graphs were plotted using Microsoft Excel 2007.

Chapter 4

Results and Discussion

4.1 Validation of analytical method

The validation of the analytical method was done to ensure that the method accurately quantifies CQAs in coffee brews and produces valid results. Sample analysis was carried out according to methodology described by Tfouni et al. (2014). Separation was achieved using C18 column as same as Tfouni et al., (2014), therefore their program was not developed in the present study due to the acceptable separation among CQAs.

Figure 7 shows the chromatogram of real sample (filter coffee) obtained using a gradient elution where we can observe the CQAs as the following order: 5-CQA, 3-CQA and 4-CQA. Under the experimental conditions, separation of CQAs was achieved during the first 30 min with isocratic elution of water (pH: 2.4)/acetonitrile, however, gradient elution was applied to clean the column and remove other interfering compounds for starting the next run. The average in Table 7 accounts for successive injections in the same day. Small variations occurred in different days. The standard was injected in the beginning to define retention time. C18-bonded silica is the most popular type of HPLC packing which is ideal for reproducible reversed phase separation in chromatography applications including acidic compounds. The term reversed-phase describes the chromatography modes namely the use of a polar mobile phase (water/acetonitrile) and a non-polar stationary phase (C18 column). In this case, different affinity of CQAs as hydrophilic compounds to the polar mobile phase resulted in the separation of compounds due to their polarity differences. Under the experimental conditions in the present study, 5-CQA being weakly retained by non-polar stationary phase and moved the fastest through the column, and eluted earliest followed by 3-CQA and 4-CQA. However some care should be taken because some differences in regarding the nomenclature of 3-CQA and 5-CQA seem to appear in several publications.

Table 7 - Average of obtained retention times for each analysed compound at 325 nm.

Analyte	*Average retention time (min)*	*CV%*
3-Caffeoylquinic acid (3-CQA)	19.97±0.49	2.44
4-Caffeoylquinic acid (4-CQA)	21.45±0.47	2.20
5-Caffeoylquinic acid (5-CQA)	8.61±0.18	2.13

Validation performance parameters like precision (repeatability and reproducibility), recovery, sensitivity as well as the limits of detection (LOD) and of quantification (LOQ) were determined with regards to 3-CQA, 4-CQA and 5-CQA.

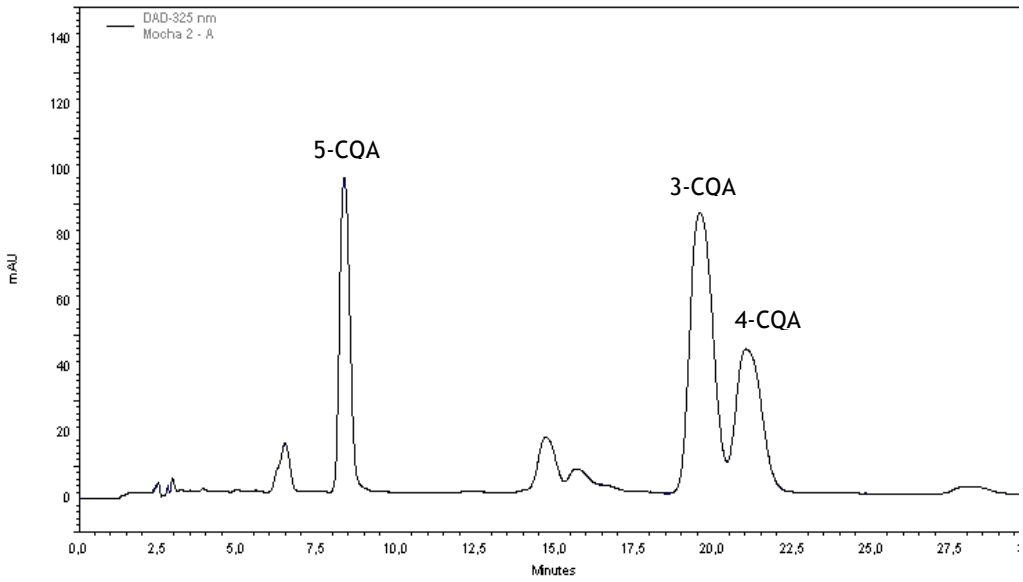


Figure 7 – Typical chromatogram of CQAs of filter coffee analyzed by HPLC-DAD at 325 nm.

4.1.1 Quantification parameters (linearity, limits of detection and quantification and sensitivity)

Calibration curves were prepared by plotting the peak area against the corresponding concentrations by injecting 10 µL of standard solutions at nine different concentration levels as follows: between 1 to 200 mg/L (1, 5, 10, 20, 40, 80, 120, 160 and 200 mg/L) for 4-CQA and 5-CQA and 2-400 mg/L (2, 10, 20, 40, 80, 160, 240, 320 and 400 mg/L) for 3-CQA. A calibration curve was constructed for each CQA (3-, 4- and 5-CQA) by duplicate injections.

Regarding the linear response of the detector, the regression lines were linear in the studied concentration range and the corresponding coefficients (R^2) of 0.999 were demonstrated over a range of 1–200 mg/L for 4-CQA and 5-CQA and a range of 2-400 mg/L for 4-CQA (Table 8 and Appendix 1). Limits of detection (LODs) and limits of quantification (LOQs) were calculated at signal-to-noise ratio of three (S/N=3) and of 10 (S/N=10), respectively. The values for LODs and LOQs were estimated using the equation (1) (Dias et al., 2010):

$$LOD, LOQ = C \times (\frac{S}{N}) \times \frac{N}{H} \qquad \textit{Equation 1}$$

where S/N is the signal/noise ratio (for LOD, S/N=3, and for LOQ, S/N=10), C=sample concentration, N=noise value when the blank is analysed, and H=value of the signal when the sample is analysed.

The LOD and LOQ of the method were clearly low as LODs of all compounds were less than the lowest concentration of the calibration curves. In case of LOQs, values were less than the

lowest concentration of the calibration curves except, 4-CQA with LOQ of 1.29 mg/L which was slightly higher than 1.00 mg/L.

The sensitivity of the method, expressed as the slope of the calibration curve, is also included in Table 8. Although, sensitivity is quite similar for the studied CQAs, the method showed higher sensitivity for 4-CQA.

Table 8 – Quantification parameters of the method for the target compounds

Analyte	*Linearity (mg/L)*	R^2	*LOD (mg/L)*	*LOQ (mg/L)*	*Sensitivity (L.mAU/mg)*
3- Caffeoylquinic acid (3-CQA)	2-400	0.999	0.37	1.24	116292
4- Caffeoylquinic acid (4-CQA)	1-200	0.999	0.39	1.29	122162
5- Caffeoylquinic acid (5-CQA)	1-200	0.999	0.18	0.58	111337

4.1.2 Reliability parameters (precision and accuracy)

Precision is a measure of the closeness of the analytical results obtained from series of replicate measurements of the same measure under the conditions of the method. Precision should be measured at different concentrations within the working range, normally at the lower, mid and upper parts. In the present study, precision was obtained for three concentration levels namely: 20, 80 and 160 mg/L for 4-CQA and 5-CQA and 40, 160 and 320 mg/L for 3-CQA.

Instrumental repeatability of the (intra-day precision) was estimated when the CQAs standards at three concentration levels (C1, C2 and C3) were analysed on the same day and six injections (Table 9, Appendix 2). Results of the intra-day precision for spiked samples are presented in (Table 11, Appendix 2). For this purpose three types of coffee, from different classes of preparation method (filter, soluble and capsule coffee) were selected and spiked at two levels. The individual results of three repeated extractions and by two injections of each spiking level are represented in (Table 11, Appendix 2). By comparing the intra-day precision, It was found that the lowest repeatability belongs to 5-CQA with 0.36% for C1, 0.17% for C2 and 0.24% for C3 (Table 9). The lowest precision of 5-CQA is probably due to the presence of the single peak at the beginning of each run in comparison to 3-CQA and 4-CQA which present subsequently but they elute nearly the same retention time so the separation was not from baseline and show small overlapped yield slight higher precision. However, all target compounds revealed acceptable precision and higher concentration of standards resulted in better precision. According to Caprioili et al. (2013), the precision of 3-CQA was higher than 5-CQA in spiked samples.

Table 9 - Average intra-day precision (%CV) of the method for the target compounds in standard solutions

Analyte	*C1**	*C2**	*C3**
3- Caffeoylquinic acid (3-CQA)	1.06	1.06	0.31
4- Caffeoylquinic acid (4-CQA)	0.95	0.80	0.54
5- Caffeoylquinic acid (5-CQA)	0.36	0.17	0.24

*Concentration of each compound in standard solutions was as follows:
C1: 3-CQA (40 mg/L), 4-CQA (20 mg/L), 5-CQA (20 mg/L);
C2: 3-CQA (160 mg/L), 4-CQA (80 mg/L), 5-CQA (80 mg/L);
C3: 3-CQA (320 mg/L), 4-CQA (160 mg/L), 5-CQA (160 mg/L).

Instrumental reproducibility (inter-day precision) was the result of the analysis of standards (C1, C2 and C3) during the three sequential days by injecting three times and the average %CV among three days for each sample was reported in Table 10 and in details in Appendix 2. All samples exhibited %CV less than 2%. In general there is a decrease in inter-day precision with increasing concentration of standards.

Table 10 – Average inter-day precision (%CV) of the method for the target compounds in standard solutions.

Analyte	*C1**	*C2**	*C3**
3- Caffeoylquinic acid (3-CQA)	0.81	0.46	1.11
4- Caffeoylquinic acid (4-CQA)	0.54	0.68	1.41
5- Caffeoylquinic acid (5-CQA)	0.36	0.23	0.32

*Concentration of each compound in standard solutions was as follows:
C1: 3-CQA (40 mg/L), 4-CQA (20 mg/L), 5-CQA (20 mg/L);
C2: 3-CQA (160 mg/L), 4-CQA (80 mg/L), 5-CQA (80 mg/L);
C3: 3-CQA (320 mg/L), 4-CQA (160 mg/L), 5-CQA (160 mg/L).

The accuracy of the analytical method was evaluated using the standard additions method and evaluating the recovery percentage. The recovery tests were performed by spiking various types of coffee brews with known quantity of the CQAs reference standards at level of C1 (80, 40 and 40 mg/L for 3-CQAs, 4-CQA and 5-CQA, respectively) and C2 (240, 120 and 120 mg/L for 3-CQAs, 4-CQA and 5-CQA, respectively), before the extraction procedure. The fortified sample was then extracted and analyzed in triplicate as described in Table 11 and Appendix 2. The average recovery (%) was reported as the mean ratio between the obtained and the expected concentrations of CQAs in fortified samples. Different coffee brews were selected based on their initial concentrations (filter, Instant and Capsule coffee) as CQAs concentration in spiked samples were within the linearity range after spiking at two concentration levels. The mean recoveries were from 91.4% to 103.3%.

As can be clearly seen, the recovery of analytes was reproducible with method precision less than 2.5%. Higher recovery was obtained from capsule coffee probably due to the higher initial concentration with regards to other brews. In general, the best recoveries results were observed for the samples spiked with level of C2 especially for 3-CQA and 4-CQA. As it was mentioned

before, these two compounds were not separated from baseline especially at higher concentrations which cause fewer recoveries than spiked samples with level of C1.

Table 11 - Intra-day precision and accuracy of coffee brews spiked at two different concentration levels

*Spiked level**	*Analyte*	*Initial Concentration (mg/L)*	*Intra-day (%)*	*Recovery (%)*
Filter coffee (Roasted and ground pure Arabica beans)				
C1	3-CQA	307.8	1.5	92.7
	4-CQA	169.7	1.5	98.3
	5-CQA	161.0	1.8	96.9
C2	3-CQA	307.8	2.0	90.4
	4-CQA	169.7	0.9	97.1
	5-CQA	161.0	1.0	97.1
Instant natural A				
C1	3-CQA	62.5	1.8	98.8
	4-CQA	51.3	1.6	100.6
	5-CQA	66.4	1.9	97.0
C2	3-CQA	62.5	1.1	91.4
	4-CQA	51.3	2.0	94.8
	5-CQA	66.4	0.5	93.9
Capsule coffee (A – type 5)				
C1	3-CQA	369.3	2.2	101.8
	4-CQA	234.9	2.2	102.5
	5-CQA	222.1	2.2	100.0
C2	3-CQA	369.3	1.6	99.8
	4-CQA	234.9	1.6	101.3
	5-CQA	222.1	1.7	103.3

*Spiked samples were prepared at two concentrations levels as follow:
C1: 3-CQA (80 mg/L), 4-CQA (40 mg/L), 5-CQA (40 mg/L);
C2: 3-CQA (240 mg/L), 4-CQA (120 mg/L), 5-CQA (120 mg/L).

4.2 CQAs content in coffee brews

For the purpose of preparing a beverage, several brewing techniques have been used. Preparation methods such as filter drip, French press, boiled and mocha and soluble coffee are the most classical patterns of brewing. Among the several brewing techniques, espresso coffee is the most appreciated coffee brew which could be prepared through automatic single-serve coffee maker (capsule and pod) or fully automatic coffee machines. To the best of the authors' knowledge, there are no comprehensive studies that compare different preparation techniques, both classical and brew prepared with new technologies and more studies were limited to typical brews. In the present study samples were divided in two groups. In the first section brews prepared with roast and ground Arabica and Robusta coffee were compared and subsequently commercial coffee brews include capsule, pod, espresso from bars, soluble coffee, iced coffee,

iced cappuccino and filter coffee prepared with commercial blends were investigated with regards to their CQAs concentration.

4.2.1 Regular roasted and ground coffee

Chlorogenic acids content present in regular roasted and ground Robusta and Arabica coffee was evaluated through quantification of CQAs in various brewing procedures including boiled, French, mocha and filter coffee. Investigations revealed the occurrence of great concentration of CQAs in all studied samples. As it can be clearly seen in Table 12, the major isomer in all samples was 3-CQA, accounting for about 50% of the total CQAs, followed by 5-CQA and 4-CQA, accounting for about 25-26% for each one, both for Robusta and Arabica coffee. Quantification of the isomers in the coffee samples was achieved by comparison of peak areas with related calibration curves. During the extraction, most of the water extractable components are washed out in the first few seconds of the extraction process but less concentration of 5-CQA than 3-CQA could be explain by this fact that 5-CQA is less water-soluble compounds than 3-CQA, yield lower concentration in the brews (Gloess et al., 2013). Although, previous literature revealed 5-CQA as the main isomer among CQAs (Fujioka et al., 2008; Mills et al., 2013; Tfouni et al., 2014; Caprioli et al., 2013) our results were in accordance to Gloess et al. (2013) who found 3-CQA at higher concentration in various types of coffee brews. Crozier et al. (2012) proved that during roasting, 3-CQA and 4-CQA being destroyed more slowly than 5-CQA. In another study (Farah et al., 2005) 5-CQA was decreased from green to light roasted degree while 3-CQA and 4-CQA increased from green beans to light roasting condition. Therefore, since different CQAs seems to have different sensitivity to various roasting conditions (Moon et al., 2009), the higher concentration of 3-CQAs than 4- or 5-CQA could be explained by the origin of the beans and their roasting degree (it is unknown for us) which could resulted in higher content of 3-CQA than 5-CQA.

In both species, when considering brewing procedure, the mocha extraction was the most efficient brewing method followed by boiled, French and filter coffee. The most influencing parameters seem to be extraction temperature and pressure because mocha extraction was performed under pressure (0.5 relative atmospheres, Peréz-Martínez et al., 2010) with hot water (probably more than 100 ºC). The decreasing order of total CQAs of samples from roast and ground Robusta in normalized mg/L basis was mocha (872.93 mg/L) > boiled (771.29 mg/L) > French (666.67 mg/L) > filter coffee (624.03 mg/L). Regarding the Arabica beans the decreasing order was similar to Robusta although the total concentration of CQAs in mocha and boiled coffee was almost the same ($p>0.05$). CQAs content in brews prepared with Arabica were as follows: 744.70 mg/L (boiled), 744.04 mg/L (mocha), 645.56 mg/L (French) and 638.58 (filter). In previous work, Peréz-Martínez et al. (2010) observed that mocha coffeemaker extracted the highest amount of CGAs per gram of ground roasted coffee, followed by the filter, and then plunger coffee makers. Within the infusion method of our study (boiled and filter coffee), boiled coffee had the greatest values for CQAs. This is consistent with the finding of Tfouni et al. (2014) who found higher content of CQAs in boiled coffee (26-295 mg/100 mL corresponding to 260-2950 mg/L) than filter coffee (24-219 mg/100 mL corresponding to 240-2190 mg/L) obtained from

beans at different roasted degree and different species. This could be due to the more contact time between roast and ground coffee and hot water during boiled extraction procedure (Tfouni et al., 2014). In the present study, filtered brews, both in Arabica and Robusta coffee, are the ones that would least contribute to CQAs intake. In other types of brews, ground coffee was extracted with hot water under pressure (mocha) or floated in hot water for a period of time (boiled and French coffee) but in case of filter brew, ground coffee was only washed out with hot water at ambient pressure without any floatation. Concerning the CQAs content of French press brew, our finding was against Gloess et al. (2013), who indicated higher extraction efficiency of 3-CQA and 5-CQA in French press coffee than mocha or even espresso coffee. This difference could be explained by different coffee/water ratio and extraction time that they used for mocha and French press brew preparation. In the present work, since all brews were prepared with coffee/water ratio of 7.5 g/100 mL, the effect of this parameter on CQAs content could be eliminated. Besides that, the degree of grinding seems to have a minor effect because, as it can be clearly seen in Figure 5 the particle size distribution in both coffee beans was almost similar.

Considering the influence of the raw material, in general, Robusta samples yielded greater CQAs content than the Arabica samples. Levels ranged from 624.03 to 872.93 mg/L for Robusta and from 638.58 mg/L to 744.70 mg/L for Arabica were detected in analysed coffee brews (Table 12). The obtained results were in accordance with the ones that have been reported by other author, where roasted and ground Robusta coffee brews contain higher chlorogenic acids levels than the Arabica ones (Tfouni et al., 2014). The statistical analysis confirms the significant differences among brews prepared with Arabica and Robusta coffee beans. The biggest difference was found in mocha coffees with concentration of 872.93 mg CQAs/L for Robusta and 744.04 mg CQAs/L for Arabica. The exception was filter coffee, where there was no remarkable difference between the values of total CQAs for Arabica (95.79 mg/L) and Robusta (93.60 mg/L) ($p>0.05$). Unlike in boiled, French press and mocha coffee brew, similar extraction percentages among CQAs of Arabica and Robusta in filter brewing process was observed although CQAs of Arabica brew (638.58 mg/L) were found slightly higher than Robusta's (624.03 mg/L). However, there was an agreement with the results of CQAs of filter coffee obtained by Ludwig et al. (2012), as sum of 3-, 4- and 5-CQAs in Arabica filter coffee (81.0 mg/100 mL corresponding to 810 mg/L), which was higher than Robusta filter coffee (56.2 mg/100 mL corresponding to 562 mg/L), although they found this difference significant.

Higher content of CGAs in Robusta coffee beans or beverages brewed with Robusta coffee were extensively reported by authors (Farah et al., 2005; Perrone et al., 2008; Ludwig et al., 2012; Tfouni et al., 2014). In some literature, lower concentration of CGAs in Arabica than Robusta coffee were found, attributed to the different coffee bean production, like wet and dry method that pronounced effects on the chemical composition of coffee seeds, especially in

water-soluble components like sugars, caffeine, trigonelline and chlorogenic acids. Generally, the wet method is used for Arabica coffee, which requires substantial amounts of water, the pulp is eliminated and it is done mucilage removal with chemical products or natural fermentation, where it is maybe the cause of a greatest loss of chlorogenic acids in comparison to the Robusta coffee that commonly uses dry method without fermentation step (Duarte et al., 2010). Nevertheless, some authors (Vignoli et al., 2011) showed that isomer 5-CQA was present in similar or higher concentrations in soluble Arabica coffee than in Robusta coffee. According to Leloup, (2006) and Clifford (1997), although green Robusta beans have a higher CGA content, these compounds seem to be more sensitive to the roasting process in a Robusta coffee matrix. Similar behaviour of Arabica and Robusta coffees suffer from a different roasting degree was also reported previously (Trugo and Macrae, 1984). These states are in agreement with data that we reported for filter coffee, where statistically the same concentration was obtained for filter Arabica and Robusta. However, the differences in other brews prepared with Arabica and Robusta could be attributed to several parameters such as coffee origins and cultivar as well as different roasting degree, which was unknown for us.

Table 12 – Caffeoylquinic acids (CQAs) content in regular roasted and ground coffee brews*

Coffee brews	*3-CQA (mg/L)*	*4-CQA (mg/L)*	*5-CQA (mg/L)*	*ΣCQAs** (mg/L)*	*ΣCQAs*** (mg/serving)*	*As a percentage of total CQA (%)*		
						3-CQA	*4-CQA*	*5-CQA*
Regular roasted ground Robusta coffee								
Boiled	365.44±6.70	199.67±3.88	206.18±3.59	771.29±1.72[b]	115.69	47.38	25.89	26.73
French	320.35±6.94	172.85±1.88	173.47±2.11	666.67±2.86[c]	100.00	48.05	25.93	26.02
Mocha	421.49±7.95	225.47±2.85	225.97±2.61	872.93±3.02[a]	52.38	48.28	25.83	25.89
Filter	296.83±12.60	162.02±3.78	165.17±3.31	624.03±5.23[d]	93.60	47.57	25.96	26.47
Regular roasted ground Arabica coffee								
Boiled	352.57±1.64	197.79±2.07	194.34±0.99	744.70±0.54[a]	111.71	47.34	26.56	26.10
French	310.97±4.05	171.48±1.17	163.12±0.99	645.56±1.71[b]	96.83	48.17	26.56	25.27
Mocha	357.25±15.59	198.47±8.31	188.32±6.30	744.04±4.89[a]	44.64	48.02	26.67	25.31
Filter	307.86±1.97	169.72±0.46	161.00±1.21	638.58±0.75[b]	95.79	48.21	26.58	25.21
Espresso lab-made	551.15±27.79	337.07±14.93	332.13±1.05	1220.35±13.37[c]	48.81	45.16	27.62	27.22

* The results correspond to the average ± standard deviation of two extraction followed by two times injection.

** In each species, values with the same letter (a, b, c or d) are not significantly different ($p>0.05$).

***Cup size for each preparation was as follows: boiled (150 mL), French (150 mL), mocha (60 mL), filter (150 mL), espresso lab-made (40 mL).

Another conclusion about the content of chlorogenic acids in coffee can be taken by the content in mg/serving, where it is considered just the quantity of the total of CQAs that will be taken in one normal single serving of coffee associated to each brewing technique. Although based on the concentration in normalized mg/L basis, mocha produced a high concentrated brew in terms of CQAs, followed by boiled, French and filter (both Arabica and Robusta) the situation was found different when content per cup size was considered. As can be seen, in Table 12 and

subsequently in Figure 8, boiled has the greatest amount of CQAs per serving (115.69 and 111.71 mg/150 mL in Robusta and Arabica, respectively), and mocha has the less content both in Robusta (52.38 mg/60 mL) and Arabica coffee (44.64 mg/60 mL). It means that a single cup of daily consumption of boiled coffee contribute to higher intake of CQAs by consumers followed by French, filter and mocha, as can be seen in Figure 8.

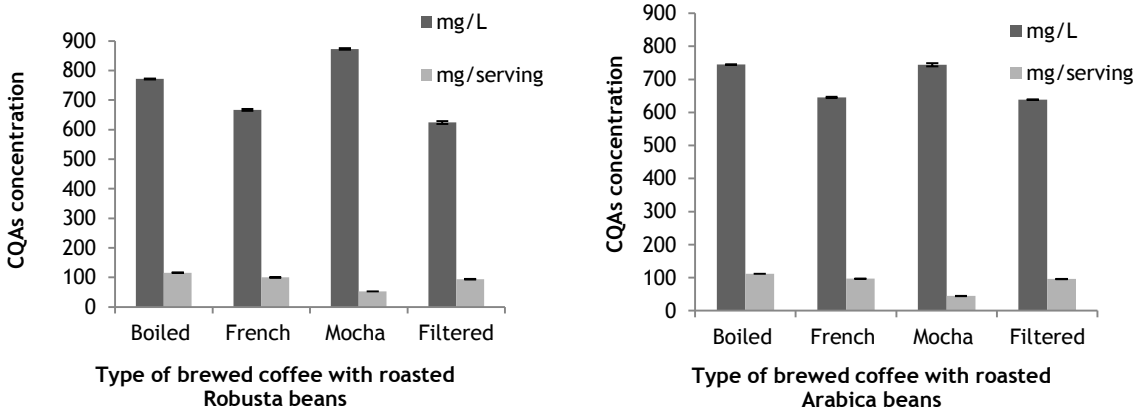


Figure 8 - Concentration of Caffeoylquinic acids (CQAs) of different coffee brews prepared with roasted Robusta and Arabica beans on both a cup and concentration basis.

4.2.2 Commercial coffee brews

In order to understand the potential variation in the amount of CQAs consumed by coffee drinkers and to go deeper into the influence of brewing techniques on concentration of phenolic compounds, various commercial coffee brews were assayed for their CQAs content in this section. As previously mentioned, the ratio of each species in the blend, roasting condition, grinding degree and origin of roasted and ground coffee used for brewing the following beverage were mostly unknown. Indeed analysis of commercial coffee brews is important because these types of brews are representative of real samples and CQAs levels commonly consumed during standard preparation outside of laboratory conditions. All available information in terms of coffee species or roasting degree supplied on the front of pack by the manufacturers has been shown previously in Table 6. This little information allowed us to approximately evaluate the extent of CQAs in brewed coffees.

For this purpose, nineteen types of commercial coffee brews prepared by different brewing techniques such as capsule, pod, espresso from bars, instant, iced coffee and iced cappuccino were selected with regards to CQAs concentration. Table 13 shows the CQAs concentration of different commercial coffee brews expressed as mg/L and contents in mg/serving. HPLC analysis of the coffee samples indicated the presence of 3-, 4- and 5-CQA in all samples. Our data

indicated that the most abundant CQAs in all considered samples (except instant natural) was 3-CQA accounting for between 36% and 50% of the total CQAs in all of the coffee samples followed by 23-35% for 5-CQA and 26 to 28% for 4-CQA. Generally speaking, the results of the processes studied varied according to the brewing mechanisms and total CQAs ranged from 1662.01 mg/L in coffee pod to 45.79 mg/L in iced cappuccino.

Regarding the CQAs content of capsules, the results were in the range of 748.40-1656.82 mg CQAs/L, much higher than those reported for classical coffee brews. Capsule A-type 1, was found to produce high concentrated brew in terms of total CQAs (1656.82 mg/L). Since all capsules were brewed with the same machine, the effect of water temperature and pressure on CQAs contents would be minimal. The information on the label of the product revealed that this is a blend of South American and East African Arabica, with a touch of Robusta, however the ratio between Arabica and Robusta as well as grinding degree are unlikely to be identical, and are known to influence CQAs content. Although, its roasting intensity is 10, but probably the effect of coffee varieties with different CQAs content and their ratio in the blend as well as other parameters like the amount of coffee powder in capsule (6.0±0.003 g/capsule) being more important than roasting intensity. Frequently, among capsules A, the lowest CQAs concentration was reported in capsule A–type 5. The degree of roasting of this capsule was at the highest value (roasting intensity of 11) which may possess more degradation of CQAs during roasting. Although the coffee quantity of capsule A-type 5 (6.13±0.13 mg/capsule) was similar to A-type1 (6.0±0.003 g/capsule), but their county of origins and coffee variety were different (capsule A-type 5 was Arabicas from Latin America and Asia) which could explain the variety of CQAs among these two types of capsules from the same brand. As it has been stated before, according to some authors, Robusta presents high levels of chlorogenic acids than Arabica coffee (Perrone et al., 2008; Tfouni et al., 2014). As it was reported previously, during roasting, chlorogenic acids content can reduce until 90% by the loss of a water molecule from the quinic acid moiety. These conclusions are in accordance with several authors (Farah et al., 2005; Fujioka & Shibamoto, 2008; Moon et al., 2009; Rodrigues & Bragagnolo, 2013). However, there are limited studies regarding CQAs content in capsule coffee (Gloess et al., 2013; Parenti et al., 2014). Gloess et al. (2013) observed 3-CQA as concentration of 15 mg/30 mL (corresponding to 500 mg/L) and found 5-CQA in lower concentration (6 mg/30 mL, corresponding to 200 mg/L) in Nespresso coffee variety of “Arpeggio”. These differences among our results and Gloess et al. (2013) could be explained by differences in raw materials and different extraction techniques for estimation of CQAs. In study of brewing procedure, Parenti et al. (2014) also measured the content of 4-CQA and 3CQA+4-CQA in capsules coffee produced with different extraction devices (Higher espresso method and Espresso system). Higher espresso method, yield 4-CQA at 0.96 mg/mL (around 960 mg/L) and the mixture of 3-and 5-CQA in amount of 2.59 mg/mL (around 2590 mg/L). The same pattern was observed in capsules extracted using espresso system for 4-CQA (0.93 mg/ mL) and 3-CQA+5-CQA (2.50 mg/mL) (Parenti et al., 2014). This discrepancy with our finding could probably be due to the lower volume of their cup (25-30 mL) which resulted in high concentrated brew. Besides that, different extraction device and the raw materials for production of capsules

may influence the CQAs concentration in brewed coffees. In general, obtained results showed capsule B has the lowest content of CQAs among all analysed capsule coffees with concentration of 748.40 mg CQAs/L. Capsule B has less quantity of coffee (5.19±0.11 g/capsule) than other capsules, which may explain the less quantity of CQAs content.

Considering normal pressure techniques, it should be stressed that the coffee bar 1 represented 3-CQA (1009.10 mg/L), 4-CQA (2098.89 mg/L) and 5 CQA (1099.29 mg/L) out of our linearity ranges. The similar behaviour was also found in coffee bar 2. We mentioned the concentration base on our calibration curves just to show the great presence of CQAs in these types of brews, although the real values of concentration may be different and should be estimated with another calibration curve with higher linearity range. Because of this reason, comparisons of CQAs of coffee bars with other brews were discarded in the present work. Table 13 shows the individual and total content of CQAs in commercial brews. Coffee pod represented the greatest content of CQAs (1662.01 mg/L), corresponding to 823.45, 436.30 and 402.30 mg/L for 3-CQA, 4-CQA and 5-CQAs, respectively. This high concentration could be attributed to the quantity of coffee per pod (7.08±0.15 g/pod). Grinding degree, ratio of Arabica to Robusta in the blend of coffee pod could also influence the extraction of CQAs, together with other technological factors like water pressure, etc. which was unknown. However, different espresso coffee machines play an important role in chemical composition of brews (Caprioli et al., 2013; Gloess et al., 2013), thus, greater CQAs content in pod could be explained by using the different extraction device. After coffee pod, the higher concentrated brews with regards to CQAs were found in vending coffee (1521.05 mg/L) and espresso coffee-lab made (1220.35 mg/L, Table 12). As previously stated in first chapter, several technological factors play an important role in the extraction of the various compounds present in ground coffee. In case of espresso-lab made, the variety of coffee (100% Arabica) may play an important role in CQAs reduction in brew. Moreover, the county of origins, roasting condition as well as type of extraction device are account as other parameters which may resulted in lower CQAs content in espresso-lab made. Although espresso coffees (capsule, pod or normal espresso) contain high content of CQAs, when consuming as a single serving size, their average content per cup would be less than classical patterns. The CQAs delivered per cup of 40 mL is maxim in capsule A-type 1 and coffee pod (average amount of 66.37 mg/40 mL) and will be minim in brewed coffee using capsule B.

CQAs content of espresso coffees has been previously investigated in the several literatures (Andueza et al., 2003; Andueza et al., 2007; Caprioli et al., 2013; Gloess et al., 2013; Parenti et al., 2014). Caprioli et al. (2013) found the most concentrated espresso coffee prepared with Arabica coffee using Aurelia EC machine. When evaluating the contents of total CQAs (3-CQA, 5-CQA, 3,5-diCQA) in Arabica espresso coffee, Arabica and Robusta accounted for 2223.4 and 2122.5 mg/L, respectively. Afterwards, Gloess et al. (2013) presented levels of 3-CQA (15 mg/30 mL or 500 mg/L) and 5-CQA (7 g/30 mL or 233 mg/L) in espresso from fully automatic machine

while with the same batch of ground coffee, different values of 3-CQA (18 mg/30 mL or 600 mg/L) and 5-CQA (8 mg/30 mL or 266 mg/L) were obtained for espresso from semi-automatic machine.

In the case of instant brewing technique, despite the other brews, the main isomer in instant natural A was 5-CQA (36%) followed by 3-CQA (34%) and 4-CQA (28%). This behaviour was in accordance with most of previous literature (Fujioka & Shibamoto, 2008; Ludwig et al., 2012; Tfouni et al., 2014). Accounting to the total CQAs, the greatest amount was obtained for Instant espresso (991.85 mg/L) followed by Instant Natural B (604.34 mg/L) and A (179.16 mg/L), the fewer amount was observed for Instant decaffeinated (171.86±1.27 mg/L). These values are in accordance to some authors which developed a study for comparison of normal coffee and decaffeinated coffee (Farah et al., 2006; Fujioka & Shibamoto, 2008) and loss of CGAs during decaffeination process was reported. In these cases, it is not possible to take many conclusions because the information about their composition and intensity are not available. These data demonstrate that when comparing commercial soluble coffee as mg/serving, they could be accounted as the potential source for delivery of remarkable amount of CQAs as instant natural B delivered 90.65 mg/150 mL which was much higher than CQAs delivered by espresso coffees in particular capsule B (29.94/40 mL). In case of commercial soluble coffees, it can be observed in Table 13 and Figure 10 that consumption of instant coffees leads to moderate intake of CQAs than classical patterns or some of the brewing produced with pressure method. However, it must be taken into account that soluble coffees suffer an additional thermal extraction treatment at high temperature after roasting which decreased their antioxidant capacity (Vignoli et al., 2011) and probably these additional processes also affected their CQAs content. Indeed, during the long hating treatment used for soluble coffee production, CGA may interact with Maillard reaction intermediates and reduced in final products (Mills et al., 2013). With regards to commercial instant coffee, Mills et al. (2013) reported the CGAs range from 37.04 mg to 121.25 mg/200 mL. The higher contents than our study are probably due to the higher consumed cup size (200 mL) in their experiment in comparison of our work (150 mL).

In general, the lowest concentration of CQAs was found in iced cappuccino (45.79 mg/L) followed by iced coffee (104.19 mg/L). Individual content of CQAs are presented in Table 13. It must be taken into account that for iced cappuccino, there are an additional process including adding other ingredients like milk and sugar which will influence the presence of CQAs in final product. According to Tfouni et al. (2014), the simultaneous consumption of milk and coffee, such as cappuccino, can produce a negative effect on CGA bioavailability which can explain the low value founded.

Table 13 - Caffeoylquinic acid content in various type of coffee brews*

Class of coffee brews	*3-CQA (mg/L)*	*4-CQA (mg/L)*	*5-CQA (mg/L)*	*ΣCQAs** (mg/L)*	*ΣCQAs*** (mg/serving)*	*As a percentage of total CQA (%)*		
						3-CQA	*4-CQA*	*5-CQA*
Capsule coffees								
Capsule A - Type 1	818.93±4.22	444.69±4.78	393.20±4.16	1656.82±0.34[a]	66.27	49.43	26.84	23.73
Capsule A - Type 2	710.01±3.77	420.11±1.85	378.94±0.66	1509.06±1.57[b]	60.36	47.05	27.84	25.11
Capsule A - Type 3	720.50±0.61	408.77±4.35	370.35±2.11	1499.63±1.88[c]	59.99	48.05	27.26	24.70
Capsule A - Type 4	604.36±5.05	349.22±2.43	318.57±1.19	1272.15±1.97[e]	50.89	47.51	27.45	25.04
Capsule A - Type 5	369.27±13.98	234.95±12.49	222.08±1.80	826.29±6.65[f]	33.05	44.69	28.43	26.88
Capsule B	356.74±11.87	200.84±1.15	190.8±1.30	748.40±6.14[g]	29.94	47.67	26.84	25.50
Capsule C	688.95±12.36	362.20±4.86	323.64±6.15	1374.78±4.61[d]	54.99	50.11	26.35	23.54
Other pressure coffees								
Vending coffee	713.64±21.24	398.39±3.57	409.02±3.61	1521.05±10.19[b]	45.63	46.92	26.19	26.89
Pod espresso	823.45±9.82	436.30±5.64	402.30±3.55	1662.01±3.19[a]	66.48	49.55	26.25	24.20
Café bar 1	1009.10±12.07	2098.89±29.41	1099.29±15.87	4207.28±9.11	105.18	23.98	49.89	26.13
Café bar 2	1181.88±7.96	683.79±6.61	704.70±6.94	2570.38±0.70	64.26	45.98	26.60	27.42
Instant coffees								
Instant natural A	62.51±2.51	51.26±0.34	65.38±1.15	179.16±1.10[c]	26.87	34.89	28.61	36.50
Instant natural B	266.70±9.58	168.52±2.23	169.11±0.99	604.34±4.64[b]	90.65	44.13	27.89	27.98
Instant decaffeinated	62.78±2.87	48.53±2.58	60.55±0.54	171.86±1.27[c]	25.78	36.53	28.24	35.23
Instant espresso	412.07±5.26	278.46±3.26	301.32±2.88	991.85±1.28[a]	49.59	41.55	28.07	30.38
Other brews*								
Filtered coffee	266.70±9.58	168.52±2.23	169.11±0.99	604.34±4.64	90.65	44.13	27.89	27.98
Iced coffee	44.51±5.21	27.82±3.38	31.85±3.50	104.19±1.02	25.00	42.72	26.71	30.57
Iced cappuccino	17.01±0.43	12.57±0.39	16.21±0.49	45.79±0.05	4.58	37.14	27.46	35.40

[*] The results correspond to the average ± standard deviation of two extraction followed by two times injection.

** In each class of brew, values with the same letter are not significantly different ($p>0.05$).

[***] Cup size for each preparation was as follows: capsules, pod, (40 mL), café bars (25 mL), instant espresso (50 mL), other instant coffees (150 mL), filter (150 mL), ice coffee (240 mL), iced cappuccino (100 mL).

**** Since these coffees were not from the same classes, statistical analysis was not done in this case.

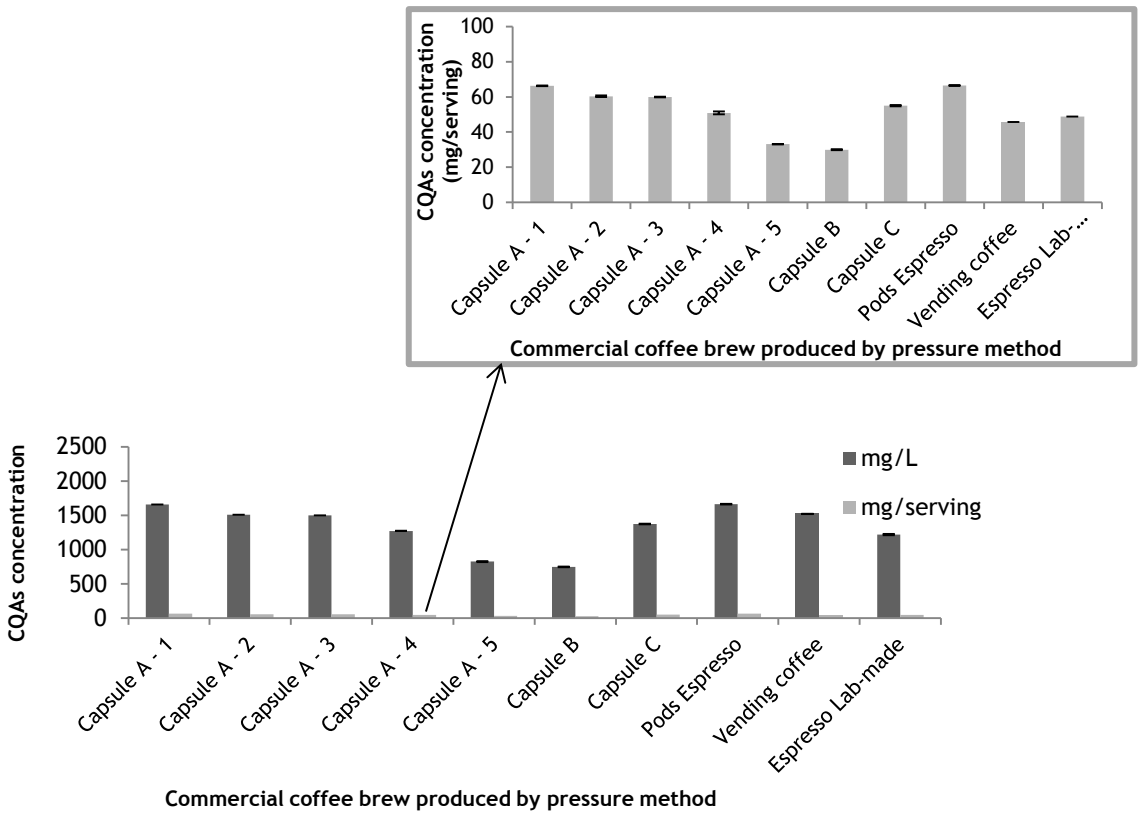


Figure 9 - Content of chlorogenic acids of different commercial coffee brews prepared under pressure. [Cup size for each preparation was as follows: capsules, pod, espresso lab made (40 mL), vending coffee (30 mL)]

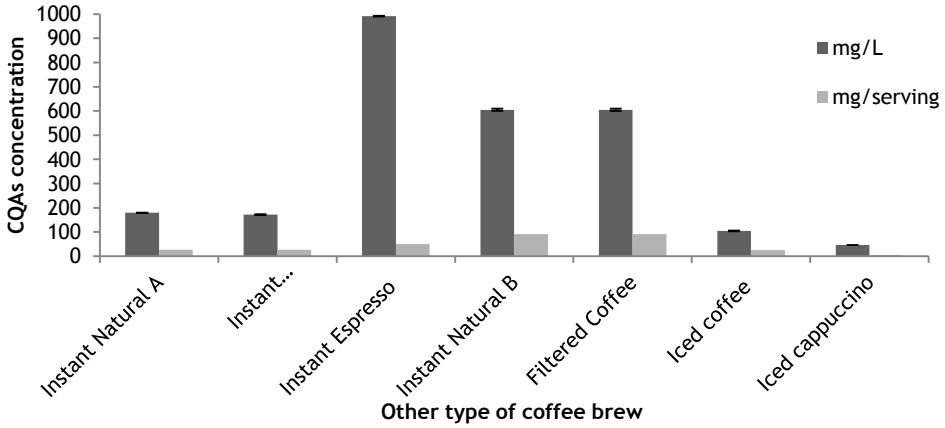


Figure 10 - Content of chlorogenic acids of different commercial coffee brews [Cup size for each preparation was as follows: instant espresso (50 mL), instant naturals and decaffeinated (150 mL), filter (150 mL), ice coffee (240 mL), iced cappuccino (100 mL)].

Conclusions

Coffee does not always have exclusively non-beneficial results and have shown also positive effects of regular coffee drinking on various aspects of health, such as psychoactive responses, neurological conditions, metabolic disorders and gonad and liver function. Coffee contains many chemicals that are affected during the roasting process. It contains caffeine, chlorogenic acid (CGA) and nicotinic acid which gives a variety of effects that is both beneficial and harmful to human beings. Chlorogenic acids (CGA) are the most abundant phenolic compounds in coffee. They are subdivided according to the nature and number of cinnamic substituents and the esterification position in the cyclohexane ring of the quinic acid. CQAs are considered as the main isomer of CGAs in coffee. In the green beans of two main cultivated coffee species, Robusta and Arabica, CGAs account for 7.0-14.4% and 4.0–8.4% of dry matter basis (dm), respectively.

Accounting for the obtained results, in the first group of the realised studies, brews prepared with roast ground Arabica and Robusta coffee, the major isomer in all samples was 3-CQA, accounting for about 50% of the total CQAs, followed by 5-CQA and 4-CQA, accounting for about 25-26% for each one, both for Robusta and Arabica coffee. In both species, when considering brewing procedure, the mocha extraction was the most efficient brewing method followed by boiled, French and filter coffee. The decreasing order of total CQAs of samples from roast and ground Robusta in normalized mg/L basis was mocha (872.93 mg/L) > boiled (771.29 mg/L) > French (666.67 mg/L) > filter coffee (624.03 mg/L) and in brews prepared with Arabica were, 744.70 mg/L (boiled), 744.04 mg/L (mocha), 645.56 mg/L (French) and 638.58 (filter). As it can be seen, Robusta coffee presents more CQAs than Arabica. For the second studied group of commercial coffee brews including capsule, pod, soluble coffee, iced coffee, iced cappuccino and filter coffee prepared with commercial blends, data indicated that the most abundant CQAs in all considered samples (except instant natural) was 3-CQA accounting for between 36% and 50% of the total CQAs in all of the coffee samples followed by 23-35% for 5-CQA and 26 to 28% for 4-CQA. The results of the processes studied varied according to the brewing mechanisms and total CQAs ranged from 1662.01 mg/L in coffee pod to 45.79 mg/L in iced cappuccino.

Since chlorogenic acids play an important role on human health, this study allows us to estimate the role of brewing techniques and type of coffee on CQAs content of brewed coffee. Since these compounds may be responsible for gastric reflux, people that suffer from this problem needs to pay more attention to the type of coffee that they intake, to assure their beverage has less chlorogenic acids contents. Besides that, our findings are useful for later equilibrating the acidity of brews for consumers who suffer from acid reflux symptoms. This

equilibration lets consumers avoid the consequences of high CGAs consumption and at the same time they intake sufficient amount for medicinal purposes.

Although good results were achieved, some limitations were faced while conducting work. This study noted a long time of each running for the analysis of each sample. An insufficient separation between the peaks of 3-CQA and 4-CQA was also verified, they didn't separate on baseline check. The isomerization of the CGAs can occur, where they may transform in each other if it was not prepared the samples one by one for each running. To prevent this, it is not possible to analyse all of the coffee samples at the same time, to not leave the samples to much time in contact with environmental temperature during the long time of analysis, preparing always new samples for each run of analysis.

As a future work, an analysis of other chlorogenic acids must be done, studying also new and recently technology of coffee production. New analytical methods to analyse many compounds in a short time in HPLC-DAD must be considered. A study about the pH of samples must be performed since a pH above 7 may destroy the chlorogenic acids, especially when the samples contain different compounds such as milk, sugar, caramel or chocolate. The effect of different kind of acids can be also studied, for preparation of mobile phase such as weak (citric or acetic acid) and strong (chloride, phosphoric or nitric acids) acids on separation of chlorogenic acids during HPLC analysis. Another future work, avoid losing chlorogenic acids during the extractions where the effect of Carrez solution to clarification of the samples should be considered and evaluated in future.

A paper based on this work was accepted by CHEMPOR and it will be published as a poster in Porto, Portugal, between the 10^{th} and 12^{th} of September, 2014. The abstract of the paper can be consulted in Appendix 3, and it was written by Marzieh Moeenfard, Lígia Rocha and Arminda Alves, entitled by "Evaluation of chlorogenic acids in coffee brews prepared by recent technologies".

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Appendix 1 - Calibration curves

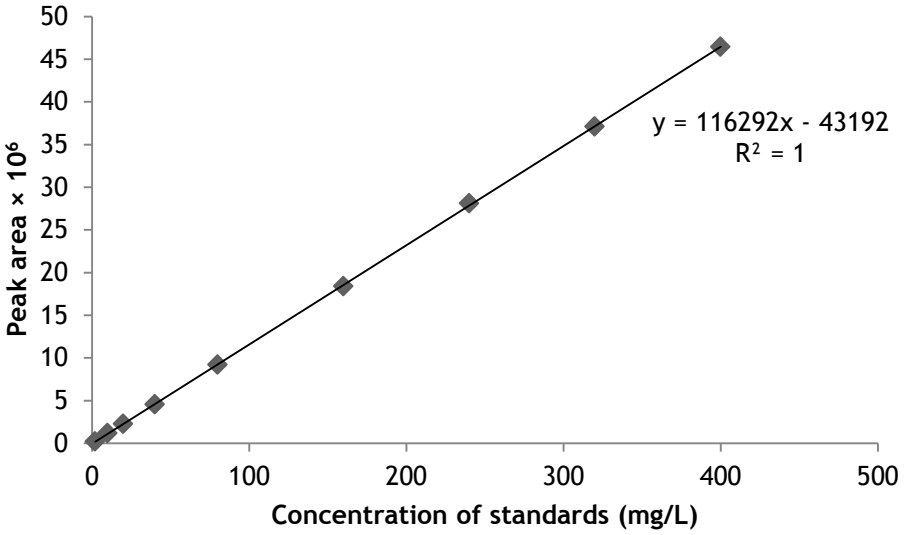


Figure A1 - Calibration curve of 3-caffeoylquinic acid (3-CQA) in linearity range of 2-400 mg/L.

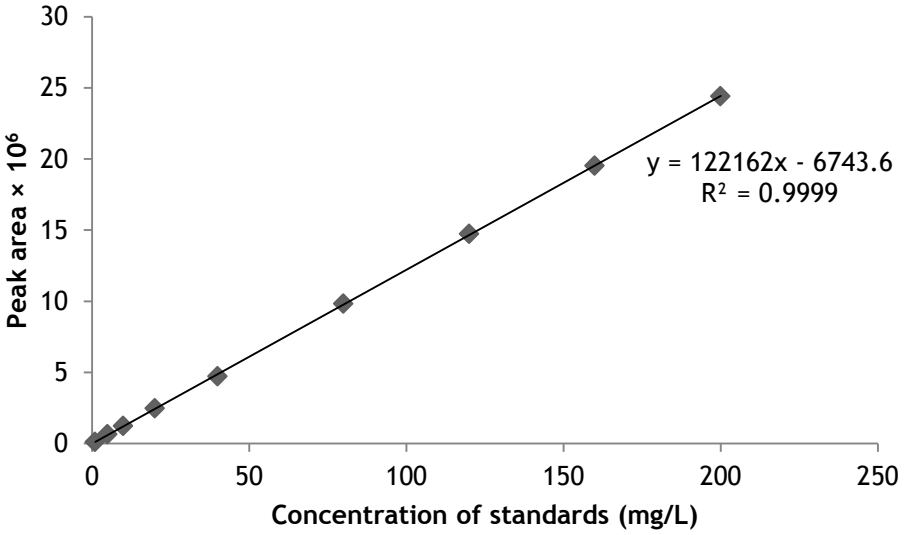


Figure A2 - Calibration curve of 4-caffeoylquinic acid (4-CQA) in linearity range of 1-200 mg/L.

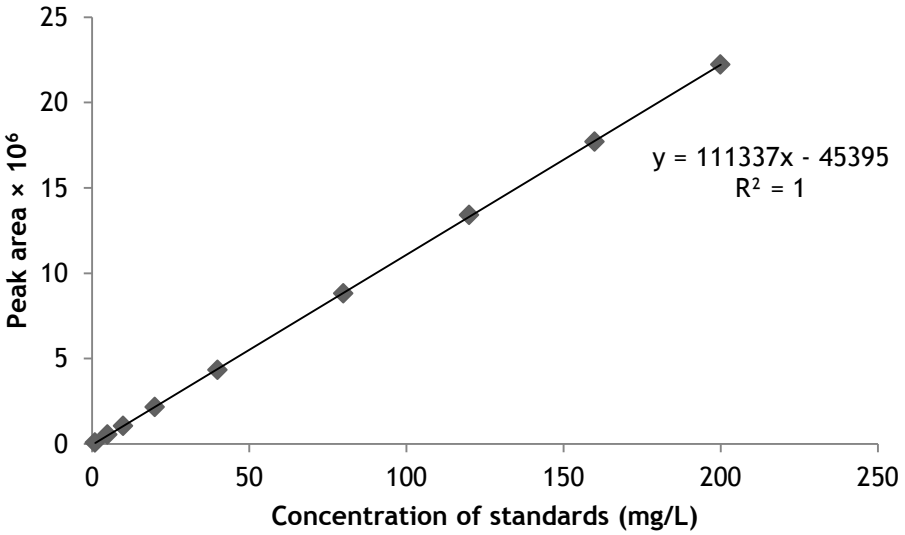


Figure A3 - Calibration curve of 5-caffeoylquinic acid (5-CQA) in linearity range of 1-200 mg/L.

Appendix 2 - Precision and Accuracy

Table 2 - Intra-day Precision (%CV) of the method for the target compounds, evaluated from calibration curves

Standards[a]	Repetition	3-CQA (mAU)	Average	%CV	4-CQA (mAU)	Average	%CV	5-CQA (mAU)	Average	%CV
C1	1	4583071	4542056	1.06	2481525	2488552	0.95	2172760	2163911	0.36
	2	4530884			2460863			2157248		
	3	4589793			2469538			2171334		
	4	4560446			2493909			2159464		
	5	4530156			2497879			2154240		
	6	4457989			2527601			2168421		
C2	1	18331694	18436117	1.06	9913710	9788080	0.80	8844764	8832423	0.17
	2	18470274			9735369			8812691		
	3	18451296			9855021			8852459		
	4	18485016			9737981			8828906		
	5	18487460			9722186			8835485		
	6	18390962			9764217			8820236		
C3	1	37024987	37041947	0.31	19628646	19586672	0.54	17708747	17744425	0.24
	2	37181767			19421612			17704942		
	3	36902811			19641024			17710790		
	4	37017163			19732166			17803725		
	5	36947042			19569238			17750306		
	6	37177912			19527348			17788040		

[a]Concentrtion of each compound in standard solutions was as follows:

C1: 3-CQA (40 mg/L), 4-CQA (20 mg/L), 5-CQA (20 mg/L);

C2: 3-CQA (160 mg/L), 4-CQA (80 mg/L), 5-CQA (80 mg/L);

C3: 3-CQA (320 mg/L), 4-CQA (160 mg/L), 5-CQA (160 mg/L).

Table 4 - Inter-day precision (%CV) of the method for the target compounds, evaluated from calibration curves

Sample[a]	Day	repetition	C1	%CV	Average	C2	%CV	Average	C3	%CV	Average
		1	4583071	0.71	0.81	18331694	0.41	0.46	37024987	0.38	1.11
	1	2	4530884			18470274			37181767		
		3	4589793			18451296			36902811		
		1	4605131	0.55		18474941	0.51		37611124	2.04	
3-CQA	2	2	4579865			18561481			37161905		
		3	4554887			18662796			36140887		
		1	4567673	1.18		18422548	0.46		37054410	0.92	
	3	2	4472703			18342424			36444280		
		3	4562057			18254606			36503716		
		1	2481525	0.42	0.54	9913710	0.92	0.68	19628646	0.63	1.41
	1	2	2460863			9735369			19421612		
		3	2469538			9855021			19641024		
		1	2393528	0.48		9641205	0.61		18850463	3.13	
4-CQA	2	2	2399720			9524656			18073485		
		3	2415868			9595305			17738579		
		1	2486747	0.71		9834711	0.51		20078197	0.47	
	3	2	2453718			9929271			20243531		
		3	2480055			9850873			20242418		
		1	2172760	0.40	0.36	8844764	0.24	0.23	17708747	0.02	0.32
	1	2	2157248			8812691			17704942		
		3	2171334			8852459			17710790		
		1	2144517	0.17		8766979	0.16		17759390	0.41	
5-CQA	2	2	2137295			8756637			17779685		
		3	2139567			8784705			17645393		
		1	2163548	0.50		8852208	0.28		17849375	0.53	
	3	2	2144864			8866868			17735542		
		3	2144974			8818600			17662666		

[a]Concentration of each compound in standard solutions was as follows:

C1: 3-CQA (40 mg/L), 4-CQA (20 mg/L), 5-CQA (20 mg/L);

C2: 3-CQA (160 mg/L), 4-CQA (80 mg/L), 5-CQA (80 mg/L);

C3: 3-CQA (320 mg/L), 4-CQA (160 mg/L), 5-CQA (160 mg/L).

Table 5 - Intra-day and accuracy of filter coffee (roasted and ground Arabica) spiked at two different concentration levels

Spiked level[a]	Analyte	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Peak area 6	Mean	%CV	Recovery (%)
C1	3-CQA	16745766	16481292	16294678	16794796	16991597	16790242	16683061	1.5	92.7
	4-CQA	9902733	9949708	9968046	10117803	10330813	10145986	10069181	1.5	98.3
	5-CQA	8472139	8540395	8473325	8677310	8830585	8781070	8629137	1.8	96.9
C2	3-CQA	23218786	22927588	22714730	23405924	22256030	23409004	22988677	2.0	90.4
	4-CQA	13621392	13593763	13825613	13708480	13895879	13786227	13738559	0.9	97.1
	5-CQA	12048170	12035081	12107930	12237140	11943644	12267483	12106575	1.0	97.1

[a]Spiked samples were prepared at two concentrations as follow:

C1: 3-CQA (80 mg/L), 4-CQA (40 mg/L), 5-CQA (40 mg/L);

C2: 3-CQA (240 mg/L), 4-CQA (120 mg/L), 5-CQA (120 mg/L).

Table 6 - Intra-day and accuracy of Instant coffee natural (Nescafé) spiked at two different concentration levels

Spiked level[a]	Analyte	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Peak area 6	Mean	%CV	Recovery (%)
C1	3-CQA	6601837	6686317	6463545	6369329	6466806	6455710	6507257	1.8	98.8
	4-CQA	4555237	4580914	4441049	4436164	4468819	4404683	4481144	1.6	100.6
	5-CQA	4603967	4628520	4459421	4423885	4475149	4468750	4509949	1.9	97.0
C2	3-CQA	12695952	12824870	13008233	12725226	12724497	12983273	12827009	1.1	91.4
	4-CQA	8013872	8117375	8021784	7840664	7673181	7894765	7926940	2.0	94.8
	5-CQA	7729655	7713960	7767205	7699592	7650791	7678791	15704775	0.5	93.9

[a]Spiked samples were prepared at two concentrations as follow:

C1: 3-CQA (80 mg/L), 4-CQA (40 mg/L), 5-CQA (40 mg/L);

C2: 3-CQA (240 mg/L), 4-CQA (120 mg/L), 5-CQA (120 mg/L).

Table 7 - Intra-day and accuracy of capsule coffee (Nespresso Darkan) spiked at two different concentration levels

Spiked level[a]	Analyte	Peak area 1	Peak area 2	Peak area 3	Peak area 4	Peak area 5	Peak area 6	Mean	%CV	Recovery (%)
C1	3-CQA	20872900	20966818	21117854	21028745	21369972	22144647	21250156	2.2	101.8
	4-CQA	13478389	13666588	13781568	13709709	13621179	14338024	13765910	2.2	102.5
	5-CQA	11375506	11477342	11541155	11585742	11654624	12121276	11625941	2.2	100.0
C2	3-CQA	28026422	28282301	27676169	27995595	28631273	28880941	28248784	1.6	99.8
	4-CQA	17723703	17549699	17472899	17074016	17939323	17632472	17565352	1.6	101.3
	5-CQA	15710126	15631956	15415302	15424896	16064269	15982103	15704775	1.7	103.3

[a]Spiked samples were prepared at two concentrations as follow:

C1: 3-CQA (80 mg/L), 4-CQA (40 mg/L), 5-CQA (40 mg/L);

C2: 3-CQA (240 mg/L), 4-CQA (120 mg/L), 5-CQA (120 mg/L);

Appendix 3 – Abstract of the Poster presentation

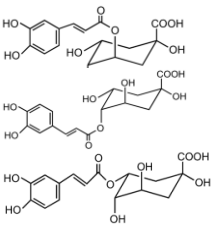
Evaluation of Chlorogenic Acids in Coffee Brews Prepared by Recent Technologies

POSTER

(#3)

Journal: **NONE**

M.Moeenfard[1], L.Rocha[1], A. Alves[1]. (1) LEPABE, Universidade do Porto, Rua Dr. Roberto Frias, Porto, Portugal; [*]aalves@fe.up.pt*



The effect of different coffee brew preparations in their chlorogenic acids (CGA) content was evaluated. The simultaneous determination of 3-caffeoylquinic acid (3-CQA), 4- caffeoylquinic acid (4-CQA) and 5-caffeoylquinic acid (5-CQA) was performed by an extraction and clean-up method (treatment with Carrez reagents I and II plus centrifugation) prior to HPLC-DAD. Fourteen coffee brews (six commercial and eight homemade) were analyzed for this purpose. Brews prepared by boiling processes and pressure methods showed higher CGA contents in comparison to instant coffee. The results revealed that normally the brews prepared with pure Robusta coffee contained more chlorogenic acids than the ones prepared with Arabica coffee.

Coffee is a complex beverage rich in large amount of chemical compounds that may contribute to biological activity [1]. Coffee bean is a source of phenolic acids which exist as a mixture of esters, ethers, or free acids [2]. Chlorogenic acids (CGA) are the main phenolic compounds in coffee which may exist as esters of trans-cinnamic acids, such as caffeic, ferulic and p-coumaric acids, with (-)-quinic acid (QA). The main classes of CGA in green coffee are caffeoylquinic acids (CQA), dicaffeoylquinic acids (diCQA) and feruloylquinic acids (FQA) [3] and are known to contribute to coffee bitterness [4]. The profile of chlorogenic acids in final coffee brew depends on coffee species, roasting and processing [5].

Substantial part of the antioxidant effect of coffee is due to the presence of chlorogenic acids [6]. Moreover they have anti-inflammatory and anticancer properties [7] and can reduce the risk of cardiovascular disease and type two diabetes [8]. Since world coffee consumption is increasing, it has recently caught more attention due to its high consumption and its subsequent impact on human health.

Most of the published papers are applied to determination of chlorogenic acids in various types of coffee beans [2] or brews regarding classical coffee consumption patterns [9,10] but little attention has been given to the quantification of these compounds in coffee brews prepared by recent technologies like capsule coffee, iced coffee or coffee pads. In recent years, due to the new portioned-machines, coffee preparation turned simplier and consequently the amount of coffee consumption may have increased. Therefore, a profound study of chlorogenic acids levels in coffee brews and resulting effects on health is necessary to be undertaken in order to clarify prevailing influence of coffee consumption on populations' health.

For this purpose the influence of brewing procedure on extraction of three caffeoylquinic acids (3-CQA, 4-CQA and 5-CQA) (Figure 1) was investigated.

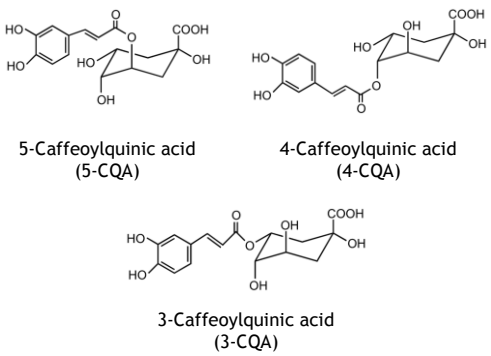


Figure1. Structures of chlorogenic acids quantified in the present study [9].

Six types of commercial coffee brews (instant, espresso and capsule coffee, coffee pad, iced coffee and coffee brew from a local vending machine) and four types of homemade coffee brews (Boiling, French press, Mocha and filter coffee) were analyzed regarding

chlorogenic acids content. Commercial coffee brews were supplied from local stores in Porto, Portugal and homemade coffees were prepared from roast and ground pure Arabica and Robusta, supplied by a local company. Three cups of coffee were prepared for each type of brew and were stored at -22 °C until analyses. Individual cup size for boiling, French, filter and instant coffee was 150 mL. For mocha and iced coffee the volume of each cup was 60 and 240 mL, respectively. Capsule, espresso, vending and coffee pad were prepared at 40 mL.

3-CQA and 4-CQA standards were purchased from Sigma-Aldrich (MO, USA) and 5-CQA was obtained from Cymit (Barcelona, Spain). The reagents used in this work were analytical or HPLC grade. Chemicals were acetonitrile and methanol (HPLC gradient grade, VWR, Belgium), zinc acetate dihydrate (VWR, Belgium) glacial acetic acid (Merck, Germany), potassium hexacyanoferrate (II) trihydrate (VWR, Belgium) and citric acid (Merck, Germany).

Carrez reagents were used for the precipitation of proteins, elimination of turbidity and breaking of emulsions. For Carrez solution I, 21.9 g of zinc acetate and 3 mL of glacial acetic acid were dissolved in distilled water and diluted in 100 mL. To prepare Carrez solution II, 10.6 g of potassium hexacyanoferrate (II) were diluted in 100 mL of distilled water [10]. Filtered water used for HPLC analysis was prepared by vacuum purification through 0.45 µm filter membranes.

Prior to extraction, three cups of coffee brews (for each type) were defrosted and mixed to reach a homogeneous mixture (40 °C). Extractions were done in triplicate according to the method of Fujioka and Shibamoto [9] with slight modification. For this purpose 3 mL of coffee was transferred to polyethylene test tube and treated with 0.1 mL of each Carrez solutions (I and II) then 0.8 mL of methanol was added and the volume was made up with distilled water to 8 mL. Thus the percentage of methanol in real samples would be 10 %. The mixture was vortexed for 1 min and let stand for 10 min. After centrifugation (4000 rpm, 10 min) and precipitation of interfering compounds, the upper phase was filtered through a 0.2 µm filter and used directly for analysis with HPLC-DAD at 325 nm.

HPLC analyses of all samples were performed in duplicate on Merck Hitachi Elite LaChromatogragh (Tokyo, Japan) with a quaternary system of pumping (L-2130) which is equipped with LiChroCART® RP-18 end-capped (250 x 4 mm, 5 µm) column attached to a guard column (4x4 mm, 5µm) of the same type and L-2200 auto sampler with L-2455 UV/vis spectrophotometry diode array detector at 325 nm. EZChrom Elite 3.1.6 software was used for data acquisition and peak integration. Peak separation was performed by gradient elution based on the analytical method of Tfouni et al., [10] with slight modification. Mobile phase composition were eluent A: 10 mM citric acid solution, acidity adjusted to pH 2.4 and eluent B: acetonitril. The gradient was programmed as follows: from 0 to 30 min 8% of B, 30 to 35 min increase to 80% of B, 35 to 40 min 80% of B, 40 to 45 min decrease to 8% of B, 45 to 50 min 8% of B. Injected volume was 10 µL. Analysis was done at flow rate of 1 mL/min at 325 nm.

Chlorogenic acids standards were prepared in aqueous solution of methanol (10 % v/v) and their dilution was done as required for calibration curves in the appropriate concentration range. Calibration curves were plotted in range of 2-200 mg/L and validation performance like precision, recovery as well as the limits of detection and quantification were obtained. Figure 2 shows the chromatogram of three chlorogenic acids in coffee from vending machine, analyzed at 325 nm.

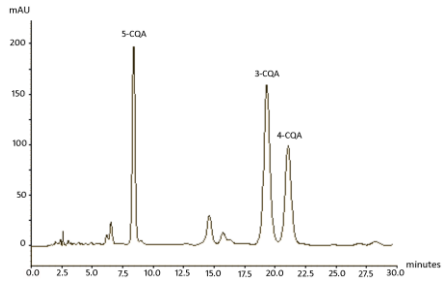


Figure 2. Typical chromatogram of filter coffee. Detection was done at 325 nm for 3-CQA, 4-CQA and 5-CQA.

Results indicated that coffee species (Arabica or Robusta) and different brewing techniques influenced the chlorogenic acids content in final coffee brews, as homemade brews prepared with Robusta coffee contained more chlorogenic acids than Arabica. Since Arabica coffees are processed by wet method, it can be considered as chlorogenic acid reduction in this species. Moreover, comparison of different brewing modes showed that more chlorogenic acids were found in coffee brew prepared under pressure and boiled coffee.

Our results indicated that consumers could receive relatively high amounts of these potentially beneficial compounds from coffee brews but acid compounds in coffee like chlorogenic acids may cause acid reflux symptoms in consumers [9]. Therefore people with acid reflux should limit drinking coffee brews from Robusta and pay attention to levels of CGA delivered per cup of coffee.

Acknowledgements
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